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Conditions stimulating neutral detergent fiber degradation by dosing branched-chain volatile fatty acids. III: Relation with solid passage rate and pH on prokaryotic fatty acid profile and community in continuous culture

Y. Roman-Garcia,[1]* K. E. Mitchell,[1] C. Lee,[2] M. T. Socha,[3] T. Park,[1]† B. A. Wenner,[1] and J. L. Firkins[1]‡
[1]Department of Animal Sciences, The Ohio State University, Columbus 43210
[2]Ohio Agricultural Research and Development Center, Wooster 44691
[3]Zinpro Corporation, Eden Prairie, MN 55344

ABSTRACT

Our objectives were to evaluate potential interactions in culture conditions that influence how exogenously dosed branched-chain VFA (BCVFA) would be recovered as elongated fatty acids (FA) or would affect bacterial populations. A 2 × 2 × 2 factorial arrangement of treatments evaluated 3 factors: (1) without versus with BCVFA (0 vs. 2 mmol/d each of isobutyrate, isovalerate, and 2-methylbutyrate; each dose was partially substituted with ^{13}C-enriched tracers before and during the collection period); (2) high versus low pH (ranging diurnally from 6.3 to 6.8 vs. 5.7 to 6.2); and (3) low versus high particulate-phase passage rate (k_p; 2.5 vs. 5.0%/h) in continuous cultures administered a 50:50 forage:concentrate diet twice daily. Samples of effluent were collected and composited before harvesting bacteria from which FA and DNA were extracted. Profiles and enrichments of FA in bacteria were evaluated by gas chromatography and isotope-ratio mass spectrometry. The ^{13}C enrichment in bacterial FA was calculated as percentage recovery of dosed ^{13}C-labeled BCVFA. Dosing BCVFA increased the even-chain *iso*-FA, preventing the reduced concentration at higher k_p and potentially as a physiological response to decreased pH. However, decreasing pH decreased recovery of ^{13}C in these even-chain FA, suggesting greater reliance on isobutyrate produced from degradation of dietary valine. The *iso*-FA were decreased, whereas *anteiso*-FA and 16:0 increased with decreasing pH. Thus, 2-methylbutyrate still appeared to be important as a precursor for *anteiso*-FA to counter the increased rigidity of bacterial membranes that had more saturated straight-chain FA when pH decreased. Provision of BCVFA stimulated the relative sequence abundance of *Fibrobacter* and *Treponema*, both of which require isobutyrate and 2-methylbutyrate. Numerous bacterial community members were shifted by low pH, including increased *Prevotella* and genera within the phylum *Proteobacteria*, at the expense of members within phylum *Firmicutes*. Because of relatively few interactions with pH and k_p, supplementation of BCVFA can stimulate neutral detergent fiber degradability via key fibrolytic bacteria across a range of conditions. Decreasing pH shifted bacterial populations and their FA composition, suggesting that further research is needed to distinguish pH from dietary changes.

Key words: passage rate, branched-chain volatile fatty acids, bacterial population, NDF degradability

INTRODUCTION

Roman-Garcia et al. (2021a,b) explained the role of branched-chain VFA (**BCVFA**) to stimulate fiber digestibility because they are required growth factors for cellulolytic bacteria; these cellulolytics also degrade hemicellulose. This benefit from adding exogenous BCVFA was expected to interact with pH and the solids passage rate ($\mathbf{k_p}$), which can be assessed independently in continuous culture. These same factors were assumed to influence lipolysis and biohydrogenation (Qiu et al., 2004; Fuentes et al., 2009). That the magnitude of time with depressed pH was more inhibitory than the actual pH per se (Cerrato-Sánchez et al., 2008) suggests a shift in bacterial physiology to overcome this stress. Response to stress has been suggested to affect the bacterial fatty acid (**FA**) profile, including those branched-

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*Current address: Cargill Animal Nutrition, Innovation Campus, Elk River, MN 55330.
†Current address: Department of Animal Science and Technology, Chung-Ang University, Anseong, Gyeonggi-do, Korea.
‡Corresponding author: firkins.1@osu.edu

chain fatty acids (**BCFA**) that were elongated from BCVFA primers (Vlaeminck et al., 2006a).

The BCFA, particularly *anteiso*-FA, have lower melting points than unbranched odd-chain and even-chain SFA (Parsons and Rock, 2013). Anaerobic bacteria have limited ability to desaturate FA or incorporate UFA and therefore rely on a mix of straight-, odd-, and branched-chain FA to maintain membrane fluidity. According to Giotis et al. (2007), a more acidic environment makes the bacterial membrane more fluid, so theoretically less BCVFA would be elongated to BCFA with decreasing pH; on the other hand, higher pH makes the bacterial membrane more rigid, and bacteria respond by incorporating more BCFA into their membrane to maintain fluidity. Vlaeminck et al. (2006a) explained that the relative concentration of primers for FA synthesis or enzyme affinity for those primers could influence BCFA profile, but they concluded that bacterial species would have a much larger influence on FA composition than environmental factors. However, ruminal bacteria can incorporate exogenous FA into their membranes in response to availability of exogenous FA and substrate (Moon and Anderson, 2001), with palmitate decreasing fluidity and oleic acid increasing fluidity. Few other studies with ruminal bacteria have evaluated FA uptake, particularly when compared with changing BCVFA availability. Some exogenous FA are stored intracellularly (Bas et al., 2003), and BCFA are still needed even when high amounts of unsaturated fat are fed (Alves et al., 2013). Thus, BCVFA elongation to BCFA is likely much more important to maintain membrane fluidity than incorporation of UFA such as oleate.

In ruminants, increasing forage in the diets increased BCFA in bacteria passing to the duodenum (Bas et al., 2003; Vlaeminck et al., 2006b), but few studies have distinguished a change in FA composition in rumen bacterial populations resulting from changing forage: concentrate independent of a change in ruminal pH. For example, Wang et al. (2018) noted that supplementation of 2-methylbutyrate to steers increased NDF degradation and certain fibrolytic bacterial rRNA gene copies more in moderate-concentrate diets than in high-concentrate diets, but starch:fiber and pH effects were indistinguishable. Before 16S rRNA gene profiling techniques, bacterial systematics often relied on profiles of specific FA (Miyagawa, 1982; Kopečný et al., 2003), whereas bacterial FA composition also likely shifts under differing ruminal conditions. Because BCVFA are precursors for bacterial BCFA in membranes and branched-chain AA (**BCAA**) in protein, BCVFA uptake likely increases with increasing bacterial growth rate, which was expected to increase with higher k_p (Roman-Garcia et al., 2021b).

The objective of this study was to test for interactions in how decreasing pH and increasing k_p affect the FA profile and recovery of dosed ^{13}C-BCVFA in bacterial FA and relate these factors to the prokaryotic community by using 16S rRNA gene sequencing. We hypothesized that increasing growth rate with high k_p should increase the percentage of BCVFA transfer into lipids of faster-growing bacteria than at low k_p. Also, we hypothesized that lower pH would decrease the elongation of BCVFA, especially 2-methylbutyrate, into *anteiso*-FA that increase membrane fluidity. Decreasing pH was projected to decrease the relative abundance of fibrolytic bacteria such as *Fibrobacter* and *Ruminococcus* spp., but BCVFA dosing was expected to help lessen this decrease, particularly when increasing k_p would increase growth pressure on bacteria.

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 × 2 × 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. All conditions and the rationale for the BCVFA treatment including 2 mmol/d each of isovalerate, isobutyrate, and 2-methylbutyrate are described in the companion study (Roman-Garcia et al., 2021b). Briefly, adding H_3PO_4 or NaOH as necessary, the low pH buffer was maintained between 6.3 and 6.4, but the high pH buffer was maintained between 6.7 and 6.8 before infusion into the fermentors, resulting in minimum pH >5.7 and >6.3, respectively. Buffer input and fluid efflux were adjusted to manipulate solids k_p while maintaining fluid k_p at 10%/h.

Doses of ^{13}C-Labeled BCVFA. Due to cost and availability, we chose [methyl-^{13}C]-2-methylbutyrate above other potential labeled products. A pilot study dosed 0, 10, or 20 mg/d of ^{13}C-labeled isoleucine (which is deaminated and decarboxylated to 2-methylbutyrate) followed by subsequent analysis of ^{13}C enrichment in FA to achieve ≥0.04 atom percentage excess in bacterial FA using techniques described subsequently (data not shown). On a molar equivalency basis, 40

mg/d of [methyl-^{13}C]-2-methylbutyrate (racemic mix) was deemed appropriate. The same dose was scaled based on carbons labeled (approximately 5 mg/d of ^{13}C for each BCVFA): 10.3 mg/d of [2,3,4-^{13}C, methyl-^{13}C]-isovalerate and 11.8 mg/d of [2,3-^{13}C, methyl-^{13}C]-isobutyrate. All isotopes were 99% enriched and purchased from Cambridge Isotope Laboratories. 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. On d 5, ^{13}C-labeled BCVFA were dosed for 4 d, which was enough for >2 turnovers of the slower solids k_p before sampling.

Bacterial Collection and Analysis for FA and Prokaryotic Community. On sampling days (d 8 to 11), whole effluent was collected at 12 h after feeding (0800 and 2000 h) on ice for each fermentor and mixed; a 30-mL sample was taken, frozen immediately, and stored at −80°C for subsequent DNA extraction. Following thawing, DNA was extracted and purified (Wenner et al., 2020) before sequencing of the 16S rRNA gene. Computational procedures were as described by Lee et al. (2021). Briefly, the Illumina MiSeq 2 × 300 paired-end protocol was used, followed by filtering, annotation, and cluster analysis using amplicon sequencing variants (**ASV**) clustered at 99% similarity. The α and β diversity indices were derived as described by Lee et al. (2021) except for Bray-Curtis dissimilarity (Wenner et al., 2020).

Another 2 subsamples of 250 mL each were taken from effluents (above) for bacterial pellet collection; the pH was brought down to 2 (monitored using a pH meter) by adding 5 *N* HCl dropwise and then stored at 4°C until particle-associated bacteria were harvested in 2 separate samples per sampling day, as described previously (Roman-Garcia et al., 2021a). One of the pellets was extracted and methylated according to Sukhija and Palmquist (1988), with the modification of Jenkins (2010) to better distinguish biohydrogenation intermediates. The FA methyl esters were analyzed as described previously (Roman-Garcia et al., 2021a) using GC, with identification using the same bacterial standard mix and the same quantification and derivation of ^{13}C enrichment using ion-ratio MS. The other pellet was used as reported in the companion paper (Roman-Garcia et al., 2021b). Background samples were taken before dosing to correct enrichments to atom percentage excess. The effluent flow of ^{13}C (corrected for background) was divided by the daily total dose of ^{13}C in the BCVFA treatments. The ^{13}C recovered in individual and total FA was derived as outlined by Roman-Garcia et al. (2021a), except that daily bacterial FA flow was used instead of FA recovered in bacterial pellets.

Statistical Analysis

Data were analyzed using PROC MIXED in SAS 9.4 (SAS Institute Inc.) according to this model:

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

where Y_{ijskl} is the dependent variable; μ is the overall population mean; B_i is the fixed effect of BCVFA dose (i = control, BCVFA); H_j is the fixed effect of pH treatment (j = high, low); K_s is the fixed effect of k_p (s = high, low); $(B \times H)_{ij}$, $(H \times K)_{js}$, $(B \times K)_{is}$, and $(B \times H \times K)_{ijs}$ are the respective interactions; F_l is the fixed effect of fermentor (l = 1 to 8); p_k is the random effect of kth period (k = 1 to 4); and ε_{ijskl} is the random error. Differences were declared at $P \leq 0.05$ and trends at $P \leq 0.10$.

RESULTS

There were no interactions among treatments for total bacterial FA flow or FA percentages in bacterial OM or DM (Table 1). However, the total FA tended ($P \leq 0.09$) to be 11 and 12% higher in bacterial OM and DM, respectively, for the main effect of dosing BCVFA. Increasing k_p decreased ($P \leq 0.02$) bacterial FA flow and FA percentage of DM by 12 and 14%, respectively.

Dosing BCVFA tended to decrease ($P = 0.07$) *iso*-13:0 percentage of total FA and tended to increase ($P = 0.08$) *iso*-14:0 and *iso*-16:0 percentages of total FA (Table 1; main effect comparison). Decreasing pH decreased ($P < 0.04$) percentage of *iso*-14:0, *iso*-15:0, and *iso*-16:0 but increased ($P = 0.01$) *anteiso*-15:0 and *iso*-17:0. Increasing k_p increased ($P = 0.01$) *iso*-15:0 and tended ($P = 0.06$) to increase *anteiso*-15:0, although this effect was most pronounced for *anteiso*-15:0 with low pH (pH × k_p interaction, $P = 0.03$). There was an interaction ($P = 0.03$) between BCVFA and k_p such that increasing k_p decreased *iso*-16:0 without dosed BCVFA but increased *iso*-16:0 percentage when BCVFA were dosed. Adding BCVFA tended to increase *anteiso*-17:0 ($P = 0.09$) and increased total even-chain *iso*-FA ($P = 0.05$) when combined with high k_p (BCVFA × k_p interactions).

Decreasing pH decreased ($P < 0.01$) 16:1 *cis*-9 and the undistinguishable FA annotated as 18:1 *cis*-9 to *cis*-12 and also *trans*-10 to *trans*-12. These overlapping peaks could not be distinguished reliably because of our short column length. The main effect of low pH increased ($P < 0.01$) 15:0, 17:0, and total odd-chain SFA compared with high pH. The percentage of 16:0

Table 1. Fatty acid (FA) profile of bacteria 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]

	Control				BCVFA										
	High pH		Low pH		High pH		Low pH			*P*-value[2]					
Item	Low k_p	High k_p	Low k_p	High k_p	Low k_p	High k_p	Low k_p	High k_p	SEM	BCVFA	pH	BCVFA × pH	k_p	BCVFA × k_p	pH × k_p
Total FA, g/d	0.793	0.630	0.805	0.633	0.722	0.679	0.850	0.768	0.102	NS	NS	NS	0.01	NS	NS
FA, % of OM	7.04	6.27	6.86	5.77	6.76	7.41	7.85	6.81	0.85	0.09	NS	NS	0.14	NS	NS
FA, % of DM	3.57	3.19	3.68	2.90	3.82	3.75	3.96	3.37	0.51	0.07	NS	NS	0.02	NS	NS
FA, g/100 g of total FA															
11:0	0.0398	0.0275	0.0621	0.0653	0.0281	0.0205	0.0548	0.0674	0.0130	NS	<0.01	NS	NS	NS	0.15
10:0 2-OH	0.00382	0.00010	0.00010	0.00237	0.00022	0.00269	0.00057	0.00188	0.00220	NS	NS	NS	NS	NS	NS
12:0	0.730	0.725	0.773	0.919	0.622	0.584	0.818	0.822	0.142	0.17	<0.01	NS	NS	NS	NS
iso-13:0	0.454	0.462	0.391	0.536	0.404	0.368	0.374	0.431	0.106	0.07	NS	NS	0.19	NS	0.09
anteiso-13:0	0.0867	0.0921	0.0802	0.0994	0.0854	0.0784	0.0753	0.0851	0.0154	NS	NS	NS	NS	NS	NS
13:0	0.323	0.311	0.391	0.399	0.353	0.330	0.335	0.384	0.038	NS	0.01	0.08	NS	NS	0.18
12:0 2-OH	0.0569	0.0510	0.0423	0.0443	0.0612	0.0397	0.0402	0.0457	0.0144	NS	0.07	NS	NS	NS	0.08
12:0 3-OH	0.0496	0.0548	0.0800	0.0824	0.0454	0.0438	0.0837	0.0958	0.0210	NS	<0.01	NS	NS	NS	NS
iso-14:0	1.20	1.23	1.08	1.00	1.49	1.45	0.84	1.41	0.20	0.08	<0.01	NS	0.16	0.11	0.16
anteiso-14:0[3]	0.0548	0.0570	0.0415	0.0382	0.0558	0.0614	0.0392	0.0534	0.0145	NS	0.01	NS	NS	NS	NS
14:0	4.42	4.43	4.05	4.74	4.15	4.21	4.27	4.13	0.45	0.20	NS	NS	NS	NS	NS
iso-15:0	3.49	4.30	2.34	3.36	3.21	3.57	2.65	3.11	0.68	NS	<0.01	NS	0.01	NS	NS
anteiso-15:0	5.15	5.24	5.61	6.41	5.08	4.83	5.36	6.45	0.78	NS	<0.01	NS	0.06	NS	0.03
15:0	4.20	3.84	5.72	4.99	5.41	4.69	4.35	5.92	0.53	0.16	0.01	0.02	NS	0.09	0.08
14:0 2-OH	0.806	0.984	1.093	0.760	0.971	0.947	0.732	0.691	0.256	NS	NS	0.16	NS	NS	0.18
14:0 3-OH	0.659	0.726	0.690	0.802	0.576	0.631	0.811	0.725	0.188	NS	0.05	NS	NS	NS	NS
iso-16:0	1.15	1.10	1.14	0.93	1.31	1.26	0.93	1.28	0.14	0.08	0.03	NS	NS	0.03	NS
anteiso-16:0[3]	0.136	0.134	0.156	0.132	0.125	0.160	0.125	0.174	0.057	NS	NS	NS	NS	0.20	NS
16:1 *cis*-9	0.464	0.492	0.328	0.350	0.440	0.625	0.245	0.381	0.071	NS	<0.01	NS	<0.01	0.04	NS
16:0	28.0	28.2	30.3	31.1	29.6	28.4	28.8	30.5	3.2	NS	0.06	NS	NS	NS	NS
iso-17:0	0.454	0.411	0.463	0.533	0.413	0.404	0.497	0.516	0.071	NS	0.01	NS	NS	NS	NS
anteiso-17:0	0.865	0.747	0.847	0.853	0.758	0.786	0.756	0.909	0.105	NS	0.20	NS	NS	0.09	0.12
17:0	0.761	0.731	0.870	0.886	0.765	0.637	0.848	0.914	0.104	NS	<0.01	NS	NS	NS	0.11
16:0 2-OH	0.0216	0.0161	0.0950	0.2964	0.0923	0.0534	0.0263	0.0872	0.0903	NS	0.20	0.13	NS	NS	NS
18:2 *cis*-9,*cis*-12	1.69	2.21	1.64	2.21	2.55	2.08	1.69	1.85	0.66	NS	NS	NS	NS	NS	NS
18:1 *cis*-9–12[4]	4.74	4.45	3.78	2.58	4.57	6.29	2.69	3.49	0.92	NS	<0.01	NS	NS	0.09	NS
18:1 *trans*-10–12[4]	8.26	9.96	7.62	6.30	7.85	10.1	7.92	5.68	1.14	NS	<0.01	NS	NS	NS	0.02
18:0	30.6	28.3	29.8	26.0	28.6	29.1	31.7	27.9	3.1	NS	NS	NS	0.12	NS	NS
iso-19:0	0.0616	0.0670	0.0643	0.0426	0.1162	0.0639	0.0851	0.0673	0.0442	NS	NS	NS	NS	NS	NS
19:0 *cis*-9[5]	0.00977	0.00000	0.02685	0.03600	0.00603	0.00000	0.00229	0.00000	0.01648	0.18	0.21	0.18	NS	NS	NS
19:0	0.0478	0.0694	0.0649	0.0371	0.0607	0.0392	0.0426	0.0412	0.0181	NS	NS	NS	NS	NS	NS
20:0	0.646	0.567	0.563	0.594	0.761	0.490	0.629	0.555	0.137	NS	NS	NS	0.06	0.19	0.15
Total unknown	5.46	5.47	6.36	6.62	5.39	5.56	5.80	6.53	1.10	NS	NS	NS	NS	NS	NS
Total OCFA[6]	5.36	4.98	7.11	6.38	6.61	5.72	5.63	7.33	0.53	NS	<0.01	0.04	NS	0.13	0.06
Total ECFA[6]	64.4	62.2	65.4	63.2	63.6	62.7	66.2	63.9	1.9	NS	0.13	NS	0.05	NS	NS
Total BCFA[7]	12.9	13.6	12.0	13.8	12.9	12.8	11.6	14.3	1.0	NS	NS	NS	0.02	NS	0.09
Total *anteiso*-FA	6.10	6.07	6.59	7.37	5.93	5.69	6.19	7.45	0.86	NS	<0.01	NS	0.07	NS	0.03
Total *iso*-FA[8]	6.82	7.56	5.48	6.41	6.94	7.12	5.38	6.81	0.87	NS	<0.01	NS	0.02	NS	NS
Total even *iso*-FA[8]	2.35	2.32	2.22	1.94	2.79	2.71	1.77	2.68	0.24	0.06	0.01	NS	NS	0.05	0.18
Total odd *iso*-FA[8]	3.95	4.71	2.80	3.90	3.62	3.97	3.15	3.63	0.73	NS	0.01	NS	0.01	NS	NS

[1]Treatments are High pH: range 6.8 to 6.3, Low pH: range 6.2 to 5.7, Low k_p: 2.5%/h, High k_p: 5.0%/h.

[2]*P*-values reported for the main effects of BCVFA dose, pH, k_p, and their 2-way interactions. All 3-way interactions were $P > 0.10$. NS = $P > 0.20$.

[3]*Anteiso*-FA are expected to be odd chain, so these are likely fatty aldehydes and are not included in sums of *anteiso*-FA or BCFA.

[4]Sum of all 18:1 FA with *cis* bonds at carbons 9 to 12, and sum of all 18:1 FA with *trans* bonds at carbons 10 to 12.

[5]19:0 *cis*-9 = *cis*-9,10-methylene octanoate.

[6]OCFA = sum of all odd straight-chain FA, ECFA = sum of all even straight-chain FA.

[7]Sum of all branched-chain FA that include *iso*-FA and *anteiso*-FA.

[8]Total *iso*-FA include all isomers, but even i*so*-FA are restricted to *iso*-14:0 + *iso*-16:0, and odd *iso*-FA are restricted to *iso*-15:0 + *iso*-17:0.

Table 2. Recovery of total ^{13}C or ^{13}C in bacterial fatty acids (FA) after dosing ^{13}C-enriched branched-chain VFA in continuous cultures maintained at either high or low pH and with either low or high solids passage rate (k_p)[1]

	High pH		Low pH			*P*-value[2]		
Item	Low k_p	High k_p	Low k_p	High k_p	SEM	pH	k_p	pH × k_p
Total ^{13}C recovered in bacterial C flow, μg/mg of ^{13}C dosed	99.9	88.8	106.4	96.7	1.0	NS	NS	NS
Total ^{13}C recovered in bacterial FA flow, μg/mg of ^{13}C dosed								
Total FA	2.93	2.72	2.20	2.13	0.93	NS	NS	NS
11:0	0.00036	0.00117	0.00028	0.00044	0.00082	NS	NS	NS
12:0	0.00121	0.00126	0.00102	0.00140	0.00047	NS	NS	NS
iso-13:0	0.0293	0.0282	0.0190	0.0331	0.0138	NS	NS	NS
anteiso-13:0	0.00457	0.00651	0.00186	0.00331	0.00135	0.06	NS	NS
13:0	0.00084	0.00106	0.00154	0.00271	0.00099	NS	NS	NS
12:0 2-OH	0.00231	0.00201	0.00128	0.00202	0.00085	NS	NS	NS
12:0 3-OH	0.00045	0.00020	0.00010	0.00086	0.00058	NS	NS	NS
iso-14:0	0.1026	0.1881	0.0453	0.0743	0.0290	0.01	0.06	NS
anteiso-14:0[3]	0.00505	0.00499	0.00085	0.00346	0.00214	NS	NS	NS
14:0	0.119	0.054	0.106	0.111	0.045	NS	NS	NS
iso-15:0	0.218	0.153	0.171	0.251	0.065	NS	NS	0.20
anteiso-15:0	0.436	0.399	0.306	0.414	0.096	NS	NS	NS
15:0	0.106	0.090	0.019	0.158	0.073	NS	NS	NS
14:0 2-OH	0.0520	0.0540	0.0134	0.0353	0.0133	0.05	NS	NS
14:0 3-OH	0.0052	0.0103	0.0192	0.0097	0.0088	NS	NS	NS
iso-16:0	0.157	0.166	0.080	0.083	0.030	0.02	NS	NS
anteiso-16:0[3]	0.0065	0.0073	0.0032	0.0071	0.0047	NS	NS	NS
16:1 *cis*-9	0.0101	0.0055	0.0116	0.0107	0.0042	NS	NS	NS
16:0	1.048	0.774	0.851	0.410	0.530	NS	NS	NS
iso-17:0	0.0129	0.0108	0.0401	0.0427	0.0098	0.01	NS	NS
anteiso-17:0	0.0517	0.0587	0.0104	0.0466	0.0146	0.11	0.15	NS
17:0	0.0172	0.0077	0.0145	0.0170	0.0114	NS	NS	NS
16:0 2-OH	0.00096	0.00017	0.00043	0.00003	0.00035	NS	0.12	NS
18:2 *cis*-9, *cis*-12	0.0214	0.0086	0.0281	0.0144	0.0128	NS	NS	NS
18:1 *cis*-9–12[4]	0.0197	0.0403	0.0080	0.0557	0.0314	NS	NS	NS
18:1 *trans*-10–12[4]	0.185	0.212	0.093	0.052	0.125	NS	NS	NS
18:0	0.301	0.281	0.365	0.230	0.322	NS	NS	NS
19:0	0.00008	0.00028	0.00003	0.00010	0.00012	NS	NS	NS
20:0	0.00057	0.00234	0.00229	0.00271	0.00139	NS	NS	NS
Total unknown FA	0.175	0.161	0.162	0.145	0.041	NS	NS	NS
Total OCFA[5]	0.131	0.109	0.031	0.173	0.075	NS	NS	NS
Total ECFA[5]	1.63	1.35	1.25	0.56	0.85	NS	NS	NS
Total BCFA[6]	1.019	1.055	0.629	0.939	0.155	0.15	NS	NS
Total *anteiso*-FA	0.491	0.462	0.321	0.464	0.099	NS	NS	NS
Total *iso*-FA[7]	0.521	0.563	0.341	0.481	0.102	NS	NS	NS
Total even *iso*-FA[7]	0.258	0.353	0.123	0.161	0.045	<0.01	0.15	NS
Total odd *iso*-FA[7]	0.253	0.163	0.253	0.338	0.088	NS	NS	0.18

[1]Treatments are High pH: range 6.8 to 6.3, Low pH: range 6.2 to 5.7, Low k_p: 2.5%/h, High k_p: 5.0%/h. Control treatment was not dosed with ^{13}C.

[2]*P*-values reported for the main effects of pH, k_p, and their 2-way interactions.

[3]*Anteiso*-FA are expected to be odd chain, so these are likely fatty aldehydes and are not included in sums of *anteiso*-FA or branched-chain FA.

[4]Sum of all 18:1 FA with *cis* bonds at carbons 9 to 12, and sum of all 18:1 FA with *trans* bonds at carbons 10 to 12.

[5]OCFA = sum of all odd straight-chain FA; ECFA = sum of all even straight-chain FA.

[6]Sum of all branched-chain FA that include *iso*-FA and *anteiso*-FA.

[7]Total *iso*-FA include all isomers, but even i*so*-FA are restricted to *iso*-14:0 + *iso*-16:0, and odd *iso*-FA are restricted to *iso*-15:0 + *iso*-17:0.

also tended ($P = 0.06$) to increase with decreasing pH. High k_p increased ($P = 0.02$) total BCFA; high k_p increased ($P = 0.02$) *iso*-FA but only tended to increase ($P = 0.07$) *anteiso*-FA. Low pH decreased ($P < 0.01$) total *iso*-FA, odd *iso*-FA, and even *iso*-FA but increased *anteiso*-FA ($P < 0.01$).

No isotope was administered to control continuous cultures because they were not dosed with BCVFA. For the BCVFA treatments, there were no effects of pH or k_p on recovery of label in total bacterial C or FA flowing to the effluent (Table 2). Low pH decreased ($P < 0.01$) ^{13}C recovery of *anteiso*-13:0 from dosed ^{13}C-labeled BCVFA. Decreasing pH decreased ($P \leq 0.01$) recovery of ^{13}C in *iso*-14:0, *iso*-16:0, and total even-chain *iso*-FA, whereas recovery in *iso*-17:0 increased ($P = 0.01$) without any change in total odd *iso*-FA. Low pH also

decreased recovery of ^{13}C in FA eluting at the same time as our *anteiso*-14:0 standards; however, as will be discussed later, this identification is questionable. Increasing k_p tended to increase ($P = 0.06$) recovery of label in *iso*-14:0.

Diversity indices were largely unaffected except for a trend ($P = 0.09$) for increasing k_p to increase Simpson's index (Table 3). The main effect of k_p or its interaction with other factors was much less common than shifts in abundance of ASV assigned at the taxonomic rank of genus resulting from the pH treatment. Nonmetric multidimensional scaling plots of the Bray-Curtis distance matrix documented that pH (Figure 1A) and period (Figure 1B) had important effects (both $P < 0.01$) on prokaryotic community structure.

Phylum *Bacteroidetes* had the highest relative percentage of ASV and had no treatment differences (Table 3). All genera within this phylum above the abundance threshold were in order *Bacteroidales*, and all but 2 of these (*S24–7* and an unclassified genus in family *Paraprevotellaceae*) were affected ($P \leq 0.10$) by the main effect of pH treatment. *Prevotella* increased markedly, as did the *CF231* genus in family *Paraprevotellaceae*, with decreasing pH. In contrast, the relative abundance of ASV assigned to a genus in family *BS11* decreased with decreasing pH. Some pH × k_p interactions were detected ($P \leq 0.08$), but the greatest shift was noted when the combination of low pH plus low k_p decreased the unclassified genus in *BS11* while increasing the unclassified genus in family *S24–7*. The unclassified genus in *RF16* was decreased ($P = 0.01$ for main effect) with increasing k_p.

The main effect of decreasing pH tended to decrease ($P = 0.06$) the relative ASV abundance assigned to phylum *Firmicutes* by an average of 14% (Table 4). Relative abundance of ASV assigned to *Pseudobutyrivibrio*, one unclassified genus in *Lachnospiraceae*, *Oscillospora*, both unclassified genera in *Ruminococcaceae*, and *Mogibacterium* decreased ($P \leq 0.09$); all were in order *Clostridiales*. Conversely, relative abundance of ASV assigned to *Shuttleworthia*, *Moryella*, and *Anaerostipes* (all *Clostridiales*) increased ($P \leq 0.03$) with decreasing pH.

Representatives of the *Veillonellaceae* family (including *Succiniclasticum*, *Dialister*, *Mitsuokella*, and 2 unclassified *Veillonellaceae* genera, all of which are in order *Clostridiales*) and *Bulleidia* (order *Erysipelotrichales*) increased with decreasing pH ($P < 0.01$ except *Dialister* and *Bulleidia*, which had trends of $P = 0.10$). Both *Butyrivibrio* and *Pseudobutyrivibrio* tended to increase ($P \leq 0.06$) with increasing k_p, whereas an unclassified *Veillonellaceae* genus and an unclassified *Christensenellaceae* genus decreased ($P \leq 0.04$). Trends ($P \leq 0.10$) for interactions were noted when increasing k_p decreased relative abundance at low pH (an unclassified genus in *Veillonellaceae* and *Clostridium*) or high pH (unclassified genus in *Mogibacteriaceae*).

Fibrobacteres and its dominant genus *Fibrobacter* tended ($P = 0.06$) to increase, and *Spirochaetes* and its dominant genus *Treponema* increased ($P = 0.01$) with the main effect of dosing BCVFA (Table 5). The benefit from BCVFA for *Fibrobacter* was more prevalent at low pH (interaction, $P = 0.08$). The relative sequence abundance of phylum *Proteobacteria* increased ($P = 0.01$) up to 15% of total ASV with decreasing pH and high k_p. The unclassified genus in *Comamonadaceae* within class β-*Proteobacteria* decreased ($P = 0.01$) with decreasing pH. *Desulfovibrio* (class δ-*Proteobacteria*) increased ($P = 0.01$) with decreasing pH. In the λ-*Proteobacteria* class, genus *Succinivibrio* had an interaction ($P = 0.03$) such that increasing k_p increased its relative ASV abundance when pH was high but not when pH was low. For the other genera in this class, the unclassified *Succinivibrionaceae* increased ($P < 0.01$) with decreasing pH. However, *Ruminobacter* and *Acinetobacter* decreased ($P \leq 0.09$) when pH decreased. Phylum *TM7* was not affected by our treatments. *Tenericutes* and some of their represented genera decreased ($P \leq 0.01$) in relative sequence abundance when pH decreased. Phylum *Verrucomicrobi*a was not affected ($P > 0.20$) by treatment. Phylum *Euryarchaeota* (in domain Archaea) and its dominant genus *Methanobrevibacte*r were also not affected ($P > 0.20$) by treatment.

DISCUSSION

2-Methylbutyrate plus either isobutyrate or isovalerate were needed to maintain the highest fiber degradability during a 24-h incubation in batch cultures of mixed ruminal microbes, but isobutyrate more consistently stimulated NDF degradability than isovalerate, as supported by literature from characterized cellulolytic bacterial strains (Roman-Garcia et al., 2021a). Dosing BCVFA tended to improve NDF digestibility by about 5.3 percentage units in the companion report (Roman-Garcia et al., 2021b). Therein, net production of isobutyrate and isovalerate decreased whereas net production of 2-methylbutyrate increased as a response to decreasing pH. We reasoned that the differences in net BCVFA accumulating inversely reflected their elongation into bacterial membranes. However, the BCFA profile could be affected both by our treatments and also by a shift in community structure toward more acid-tolerant amylolytics, as reported in other studies (Vlaeminck et al., 2006a). We are among the first to evaluate the mode of action of BCVFA using both ^{13}C-BCVFA dosing and the bacterial community.

Table 3. Bacterial diversity and relative abundance (% of total amplicon sequence variants, ASV) of genera within phylum *Bacteroidetes* 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]

	Control				BCVFA										
	High pH		Low pH		High pH		Low pH			*P*-value[2]					
Item	Low k_p	High k_p	Low k_p	High k_p	Low k_p	High k_p	Low k_p	High k_p	SEM	BCVFA	pH	BCVFA × pH	k_p	BCVFA × k_p	pH × k_p
Diversity indices															
ASV, n	1,448	1,192	1,267	1,247	1,263	1,301	1,534	1,210	150	NS	NS	NS	0.18	NS	NS
Chao1	1,535	1,244	1,340	1,319	1,366	1,385	1,668	1,277	174	NS	NS	NS	0.16	NS	NS
Evenness	0.770	0.806	0.801	0.793	0.775	0.781	0.801	0.793	0.022	NS	NS	NS	0.61	NS	NS
Faith's phylogenetic diversity	64.8	57.9	59.7	57.9	60.6	61.4	64.7	57.4	3.5	NS	NS	NS	0.11	NS	NS
Shannon's index	8.07	8.23	8.25	8.13	7.94	8.08	8.48	8.11	0.293	NS	NS	NS	0.77	NS	NS
Simpson's index	0.972	0.987	0.974	0.979	0.977	0.982	0.977	0.986	0.008	NS	NS	NS	0.09	NS	NS
Good's coverage	0.995	0.997	0.996	0.996	0.996	0.996	0.994	0.996	0.001	NS	NS	NS	0.18	NS	NS
Bacteroidetes	53.2	49.2	50.8	47.1	49.8	47.4	46.1	47.1	2.8	NS	NS	NS	NS	NS	NS
Prevotella	13.8	13.0	25.5	26.6	16.2	15.7	25.8	26.6	3.2	NS	<0.01	NS	NS	NS	NS
UN (*Prevotellaceae*)[3]	1.092	0.923	1.216	0.710	1.305	0.827	0.232	0.710	0.322	NS	0.10	0.15	NS	NS	NS
UN (*Paraprevotellaceae*)	1.86	1.52	1.12	0.98	1.92	2.02	1.32	0.98	0.27	NS	<0.01	NS	NS	NS	NS
YRC22 (*Paraprevotellaceae*)	1.45	2.16	1.37	1.32	1.29	1.71	1.41	1.32	0.29	NS	0.10	NS	NS	NS	0.08
CF231 (*Paraprevotellaceae*)	0.664	0.616	1.565	0.802	0.799	0.782	1.356	0.802	0.242	NS	0.01	NS	0.11	NS	0.17
UN (*BS11 Gut Group*)	16.06	9.40	2.33	3.42	10.35	9.24	0.95	3.42	1.91	0.18	<0.01	NS	NS	0.16	0.03
UN (*S24–7*)	4.45	7.13	6.37	3.54	5.10	6.51	4.87	3.54	1.15	NS	NS	NS	NS	NS	0.07
BF311 (*Bacteroidaceae*)	0.665	0.535	0.334	0.202	0.641	0.552	0.183	0.202	0.114	NS	<0.01	NS	0.19	NS	NS
UN (*RF16*)	0.551	0.325	0.343	0.214	0.369	0.285	0.339	0.214	0.100	NS	0.06	NS	0.01	NS	NS
UN (*Bacteroidales*)	9.1	10.2	9.1	7.5	8.7	7.7	7.4	7.5	1.9	NS	NS	NS	NS	NS	NS
UN (*Bacteroidales*)	3.19	3.00	1.28	1.55	2.68	1.72	1.97	1.55	0.52	NS	0.01	0.08	NS	NS	NS

[1]Treatments are High pH: range 6.8 to 6.3, Low pH: range 6.2 to 5.7, Low k_p: 2.5%/h, High k_p: 5.0%/h.

[2]*P*-values reported for the main effects of BCVFA dose, pH, k_p, and their 2-way interactions. All 3-way interactions were $P > 0.10$. NS = $P > 0.20$.

[3]UN = unclassified genus (lowest classified taxon within parentheses).

BCVFA Effects on General Bacterial Fatty Acid Metabolism

The total FA flow in bacteria averaged about 1.0 g/d (Table 1), which is about 15% of the total bacterial CP flow (Roman-Garcia et al., 2021b). The decreased bacterial FA flow with increasing k_p could be a result of dilution of dietary FA that adsorb to bacterial cells or small particles that contaminate harvested bacteria. If increased concentration of BCVFA (by dosing) would increase substrate-level competition as primers in the fatty acid synthetase system, then we would expect an increased representation of BCFA in the total bacterial FA. However, bacterial FA flow was not increased statistically (numerical increase of 5.6%), even though dosing BCVFA tended to increase total bacterial FA by 11% of OM while having no effect on the BCFA percentage of total FA (Table 1). We noted similar increases in FA concentration when BCAA or BCVFA were dosed to batch cultures (Roman-Garcia et al., 2021a). Robinson and Allison (1969) reported that increasing dose of 2-methylbutyrate increased its incorporation into isoleucine in *Prevotella ruminicola* with respect to transfer to lipid. They noted varying recoveries of ^{14}C from 2-methylbutyrate in protein versus lipid fractions from several strains of ruminal bacteria and one methanogen. Because there was a very large difference between *Ruminococcus albus* and *Ruminococcus flavefaciens* (88 vs. 5% label recovery in lipid) despite similar taxonomy and niche, BCVFA incorporation into protein versus lipid fractions likely depends on needs for growth relative to precursor availability and any potential shifts in the FA:AA ratio in cells under those conditions.

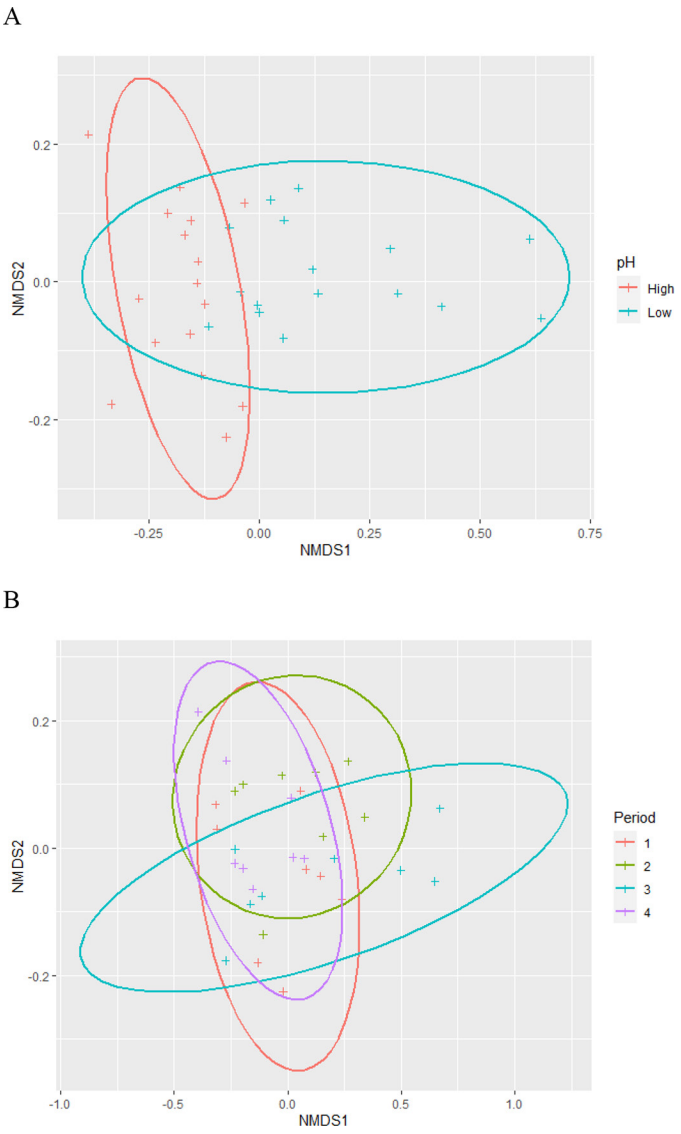

Figure 1. Bray-Curtis plots (nonmetric multidimensional scaling, NMDS axes) of a Bray-Curtis distance matrix for 16S rRNA gene relative abundance (rarefied data) from continuous cultures dosed with branched-chain VFA ($P > 0.20$) and maintained with high or low particulate passage rate ($P > 0.20$) and high or low pH ($P < 0.01$; panel A). The effect of incubation period (1–4) was $P < 0.01$ (panel B). There were no interactions ($P > 0.20$) among treatments.

Although little effect of imbalance was noted among individual BCVFA/BCAA pairs (Allison et al., 1984; Atasoglu et al., 2004), imbalance can occur among BCAA (Kajikawa et al., 2005). All 3 of the BCAA are synthesized from pyruvate or a derivative thereof, such as threonine as a precursor for isoleucine (Kaiser and Heinrichs, 2018). Relatively little is known regarding the regulatory control of FA synthesis in gut anaerobic bacteria, although those facultative anaerobes studied appear to control their lipid:protein ratio under differing growth conditions (Parsons and Rock, 2013). The latter authors noted that starvation of leucine and isoleucine in cultures changed expression of genes involved in FA synthesis and membrane fluidity in gram-positive *Bacillus subtilis* and gram-negative *Escherichia coli*, respectively. Thus, increased total FA concentration resulting from dosing BCVFA in our study might result from stimulating FA synthesis in some unknown way, whereas we assume that imbalance is much less likely with provision of BCVFA compared with BCAA. Moreover, substituting BCVFA for BCAA increased recovery of dose in bacterial FA harvested from mixed ruminal microbes in vitro (Roman-Garcia et al., 2021a).

Branched-Chain FA Profile and Recovery of ^{13}C from BCVFA Doses

Recovery of label in AA was not assayed in the present experiment but must have been high because we recovered about 100 μg/mg of ^{13}C-BCVFA dosed (about

Table 4. Relative abundance (% of total amplicon sequence variants) of bacterial genera within the phylum *Firmicutes* 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]

	Control				BCVFA					*P*-value[2]					
	High pH		Low pH		High pH		Low pH								
Item	Low k_p	High k_p	Low k_p	High k_p	Low k_p	High k_p	Low k_p	High k_p	SEM	BCVFA	pH	BCVFA × pH	k_p	BCVFA × k_p	pH × k_p
Firmicutes	34.5	34.5	26.8	33.1	35.3	32.7	28.8	28.5	3.5	NS	0.06	NS	NS	NS	NS
Butyrivibrio	2.65	3.29	2.00	2.68	2.46	2.38	1.94	3.11	0.49	NS	NS	NS	0.06	NS	NS
Pseudobutyrivibrio	1.66	2.06	0.49	1.47	1.56	1.81	0.86	0.94	0.28	NS	<0.01	NS	0.03	0.19	NS
Coprococcus	1.236	1.469	0.683	1.207	0.691	0.824	0.931	0.893	0.293	0.16	NS	0.17	NS	NS	NS
Shuttleworthia	0.409	0.423	0.767	0.898	0.209	0.436	1.445	0.596	0.259	NS	0.01	NS	NS	NS	0.18
Moryella	0.106	0.077	0.144	0.146	0.110	0.108	0.213	0.198	0.035	0.15	0.01	NS	NS	NS	NS
Anaerostipes	0.058	0.052	0.302	0.159	0.089	0.118	0.122	0.159	0.063	NS	0.03	0.14	NS	NS	NS
UN (*Lachnospiraceae*)[3]	3.79	5.85	1.85	2.77	3.49	5.52	0.95	1.46	1.54	NS	0.02	NS	NS	NS	NS
UN (*Lachnospiraceae*)	1.30	1.74	1.37	1.42	1.93	1.41	1.18	1.15	0.34	NS	NS	NS	NS	NS	NS
Ruminococcus	2.25	2.85	2.75	2.41	2.28	2.80	3.12	2.60	0.45	NS	NS	NS	NS	NS	0.12
Oscillospira	0.611	0.428	0.207	0.188	0.313	0.465	0.136	0.159	0.089	0.20	<0.01	NS	NS	0.17	NS
UN (*Ruminococcaceae*)	2.60	2.19	2.28	2.12	2.72	2.30	1.64	1.79	0.43	NS	0.09	NS	NS	NS	NS
UN (*Ruminococcaceae*)	0.918	0.681	0.611	0.542	0.756	0.655	0.492	0.558	0.150	NS	0.04	NS	NS	NS	NS
Succiniclasticum	1.564	1.291	2.061	2.511	1.651	1.778	1.996	1.797	0.329	NS	0.04	0.17	NS	NS	NS
Dialister[4]	<0.001	<0.001	0.041	0.585	<0.001	<0.001	0.133	0.291	0.232	NS	0.10	NS	NS	NS	NS
Mitsuokella[4]	<0.001	<0.001	0.210	0.187	<0.001	<0.001	0.413	0.190	0.068	NS	<0.01	NS	0.18	NS	0.18
UN (*Veillonellaceae*)	0.080	0.056	0.388	0.340	0.076	0.071	0.485	0.277	0.057	NS	<0.01	NS	0.04	NS	0.10
UN (*Veillonellaceae*)[4]	0.013	0.000	0.142	0.287	0.037	0.028	0.214	0.247	0.075	NS	<0.01	NS	NS	NS	NS
Clostridium	0.626	0.811	0.839	0.680	0.774	0.748	1.116	0.778	0.126	NS	NS	NS	NS	NS	0.09
UN (*Christensenellaceae*)[4]	4.58	2.90	0.65	0.94	6.35	3.25	0.22	0.91	1.43	NS	NS	NS	<0.01	NS	NS
UN (*Mogibacteriaceae*)	0.630	0.456	0.565	0.517	0.527	0.470	0.358	0.564	0.099	NS	NS	NS	NS	0.12	0.09
Mogibacterium	0.141	0.093	0.119	0.056	0.120	0.080	0.028	0.041	0.038	NS	0.07	NS	0.16	NS	NS
UN (*Clostridiales*)	4.89	4.49	5.20	4.86	4.72	3.86	6.13	4.68	0.76	NS	0.16	NS	0.13	NS	NS
UN (*Clostridiales*)	2.97	2.80	2.59	3.59	2.96	3.01	2.65	2.90	0.37	NS	NS	NS	NS	NS	NS
Rummeliibacillus[4]	0.078	0.022	0.048	0.441	0.082	0.136	0.137	0.537	0.195	NS	0.12	NS	0.15	NS	0.14
Bulleidia	0.055	0.031	0.083	0.168	0.015	0.018	0.007	0.280	0.088	NS	0.10	NS	0.16	NS	0.13
Sharpea[4]	0.000	0.000	0.010	0.221	0.006	0.000	0.057	0.380	0.154	NS	0.12	NS	NS	NS	0.20

[1]Treatments are High pH: range 6.8 to 6.3, Low pH: range 6.2 to 5.7, Low k_p: 2.5%/h, High k_p: 5.0%/h.

[2]*P*-values reported for the main effects of BCVFA dose, pH, k_p, and their 2-way interactions. Only *Shuttleworthia* had a tendency for a 3-way interaction ($P = 0.10$). All other 3-way interactions were $P > 0.10$. NS = $P > 0.20$.

[3]UN = unclassified genus (lowest classified taxon within parentheses).

[4]Fermentor effect not included in the model to avoid negative values. See Statistical Analysis section in the Materials and Methods.

Table 5. Relative abundance (% of total amplicon sequence variants) of bacterial genera within other phyla (other than *Bacteroidetes* and *Firmicutes*) and the main archaeal phylum and genus 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]

	Control				BCVFA										
	High pH		Low pH		High pH		Low pH			*P*-value[2]					
Item	Low k_p	High k_p	Low k_p	High k_p	Low k_p	High k_p	Low k_p	High k_p	SEM	BCVFA	pH	BCVFA × pH	k_p	BCVFA × k_p	pH × k_p
Fibrobacteres	0.252	0.328	0.156	0.229	0.282	0.335	0.501	0.356	0.082	0.06	NS	0.08	NS	NS	NS
Fibrobacter	0.252	0.328	0.156	0.229	0.282	0.335	0.501	0.356	0.082	0.06	NS	0.08	NS	NS	NS
Spirochaetes	2.90	2.40	5.70	2.50	6.50	6.22	6.08	7.03	1.45	0.01	NS	NS	NS	NS	NS
Treponema	1.90	1.87	4.71	2.19	5.73	4.98	5.87	6.16	1.28	0.01	NS	NS	NS	NS	NS
Sphaerochaeta	0.353	0.117	0.161	0.112	0.231	0.357	0.166	0.172	0.100	NS	0.14	NS	NS	0.18	NS
UN (*Spirochaetes*)[3]	0.558	0.401	0.794	0.145	0.499	0.879	0.023	0.673	0.485	NS	NS	NS	NS	0.09	NS
Proteobacteria	5.2	10.0	12.8	12.1	5.3	10.0	15.1	11.0	3.1	NS	0.01	NS	NS	NS	0.07
UN (*Comamonadaceae*)[4]	0.687	1.289	0.035	0.082	0.684	1.350	0.060	0.112	0.339	NS	<0.01	NS	0.14	NS	NS
Desulfovibrio	0.163	0.118	0.427	0.538	0.076	0.057	0.354	0.711	0.180	NS	<0.01	NS	NS	NS	NS
Succinivibrio	0.33	3.84	1.47	1.59	1.41	4.80	1.06	1.47	1.11	NS	0.09	NS	0.01	NS	0.03
UN (*Succinivibrionaceae*)	2.23	3.01	9.94	9.37	1.75	1.51	13.11	7.33	3.13	NS	<0.01	NS	NS	NS	0.33
Ruminobacter	0.708	0.468	0.032	0.098	0.317	0.853	0.176	0.519	0.263	NS	0.06	NS	NS	0.19	NS
Acinetobacter	0.201	0.329	0.152	0.031	0.271	0.285	0.070	0.322	0.121	NS	0.09	NS	NS	NS	NS
Tenericutes	1.75	1.89	1.22	0.88	1.27	2.06	1.12	0.91	0.25	NS	<0.01	NS	NS	NS	0.03
Anaeroplasma	0.651	0.540	0.285	0.165	0.440	0.561	0.044	0.128	0.126	0.20	<0.01	NS	NS	NS	NS
UN (RF39)	0.582	0.600	0.623	0.637	0.520	0.615	1.030	0.649	0.168	NS	0.19	NS	NS	NS	NS
UN (ML615J-28)	0.319	0.604	0.275	0.047	0.210	0.770	0.018	0.093	0.177	NS	0.01	NS	0.17	NS	0.06
Actinobacteria[4]	0.06	0.03	0.71	1.75	0.04	0.03	0.38	1.97	0.79	NS	0.03	NS	0.20	NS	0.19
Bifidobacterium[4]	<0.01	<0.01	0.64	1.68	<0.01	<0.01	0.29	1.81	0.76	NS	0.03	NS	0.20	NS	0.20
TM7	0.481	0.501	0.489	0.643	0.524	0.582	0.797	0.667	0.244	NS	NS	NS	NS	NS	NS
UN (F16)	0.479	0.501	0.486	0.642	0.522	0.581	0.792	0.665	0.245	NS	NS	NS	NS	NS	NS
Verrucomicrobia	0.348	0.391	0.241	0.241	0.218	0.318	0.254	0.274	0.074	NS	NS	NS	NS	NS	NS
UN (RFP12)	0.307	0.309	0.217	0.207	0.187	0.267	0.210	0.243	0.067	NS	NS	NS	NS	NS	NS
Euryarchaeota	0.169	0.094	0.150	0.150	0.212	0.142	0.191	0.181	0.055	NS	NS	NS	NS	NS	NS
Methanobrevibacter	0.144	0.085	0.145	0.132	0.183	0.122	0.169	0.159	0.051	NS	NS	NS	NS	NS	NS

[1]Treatments are High pH: range 6.8 to 6.3, Low pH: range 6.2 to 5.7, Low kp: 2.5%/h, High kp: 5.0%/h.

[2]*P*-values reported for the main effects of BCVFA dose, pH, k_p, and their 2-way interactions. Only *Acinetobacter* had a tendency for a 3-way interaction ($P = 0.10$). All other 3-way interactions were $P > 0.10$. NS = $P > 0.20$.

[3]UN = unclassified genus (lowest classified taxon within parentheses).

[4]Fermentor effect not included in the model to avoid negative values. See Statistical Analysis section in the Materials and Methods.

10%), but <3% of that total recovered ^{13}C was in total FA (Table 2). Roman-Garcia et al. (2021a) already justified why recovery should be higher in BCAA and why a numerically low recovery of ^{13}C in FA is still biologically meaningful. Although the ^{13}C enrichment was much higher in BCFA than in other FA in our study (data not shown), low enrichment in more abundant FA such as 16:0 (Table 2) suggests considerable labeling of acetyl-CoA derived from dosed ^{13}C-BCVFA. *Clostridium sporogenes* interconverted radiolabeled valine with that of leucine, and leucine conversion to valine yielded acetate in their proposed pathway (Monticello and Costilow, 1982). Degradation of BCVFA-CoA (originally from BCAA) to acetyl-CoA or propionyl-CoA is documented in several nonrumen *Proteobacteria* (Kazakov et al., 2009). Any metabolism of BCVFA to acetate or acetyl-CoA would allow distribution of ^{13}C from our BCVFA into many compounds, especially bacterial FA (Kristensen, 2001). Unfortunately, deconvolution analysis of isotopomers (Atasoglu et al., 2004) was not possible with isotope-ratio MS, so these findings need further corroboration.

Even-chain *iso*-FA percentages in total FA were affected by BCFA, pH, and BCVFA × k_p, but results were mostly due to decreasing even-chain *iso*-FA when BCVFA was combined with low pH at low k_p (Table 1). In contrast to generally higher total concentrations, the recovery of ^{13}C in even-chain *iso*-FA decreased markedly with decreasing pH (Table 2). The ^{13}C recovery in *anteiso*-13:0 decreased in the low pH treatments, but it was a low percentage of total *anteiso*-FA recovery, which was not affected by pH. That total *anteiso*-FA was only decreased by low pH when k_p was low (Table 1) suggests that the supply of 2-methylbutyrate is important in rapidly growing bacteria, regardless of pH. The lower pH shifted relative abundance of many bacteria, notably increasing *Prevotella*, *Veillonellaceae*, and *Succinivibrionaceae* (Tables 3 to 5). These bacteria are not known to require BCVFA, and *Prevotella ruminicola*, *Megasphaera elsdenii* (family *Veillonellaceae*), and *Ruminobacter amylophilus* (family *Succinivibrionaceae*) are characterized proteolytics (Stewart et al., 1997). A sequenced strain of *Succinivibrio dextrinosolvens* was suggested to grow on BCAA (Hailemariam et al., 2020). Therefore, more BCAA from RDP (which is obviously unlabeled) might have been decarboxylated and inserted into membranes, resulting in less mixing with extracellularly dosed ^{13}C-BCVFA.

There are 2 potential explanations for why recovery of ^{13}C was not affected for odd-chain *iso*-FA but decreased with decreasing pH for even-chain *iso*-FA. First, many *Proteobacteria* degrade isovaleryl-CoA with a different enzyme complex to acetyl-CoA than that used to degrade the CoA esters of isobutyrate and 2-methylbutyrate (Kazakov et al., 2009). Isobutyrate might have been catabolized more than isovalerate by the increasing members of *Proteobacteria* in our low pH treatments. Second, isovalerate could be metabolized differently in *Prevotella*, which also increased with decreasing pH. Dosing isovalerate profoundly increased expression of BCAA synthetic enzymes, perhaps as a stress response, when compared with isobutyrate (2-methylbutyrate was not tested) in *Prevotella bryantii* B14 (Trautmann et al., 2020). In that study, isobutyrate increased even-chain *iso*-FA at the expense of *anteiso*-FA and linear-chain FA, whereas isovalerate had a minimal effect on FA profile. *Prevotella* spp. contribute substantially (often predominantly) to proteolysis in the rumen (Hartinger et al., 2018). *Prevotella* spp. hydrolyze protein followed by transport of small peptides for intracellular deamination, yet they regulate ammonia assimilation enzymes as needed (Kim et al., 2017); they probably shift more to peptides as fuel as carbohydrate becomes depleted. Leucine (pairing with isovalerate) was metabolized to a greater extent than the other 2 BCAA in batch cultures of mixed ruminal bacteria (Atasoglu et al., 2004). Thus, decreasing net production of isobutyrate and isovalerate relative to 2-methybutyrate (Roman-Garcia et al., 2021b) is likely explained by increasing catabolism, thus explaining decreasing recovery of ^{13}C (mostly or entirely from isobutyrate) in even-chain *iso*-FA (Table 2) even though the profile was not markedly reduced (Table 1).

Decreased pH tended to increase *anteiso*-17:0 and total *anteiso*-FA in bacteria harvested from continuous cultures (Berthelot et al., 2019). In our study, low pH tended to increase the 16:0 percentage of bacterial FA, and pH interacted with both BCVFA and k_p for OCFA such that both OCFA and *anteiso*-FA tended to be increased the most by low pH when k_p was high. *Bacillus subtilis* inserts 15:0 and *anteiso*-FA primarily in the *sn*-1 and *sn*-2 positions, respectively, of its membrane's phospholipids (Kaneda, 1991). Although little is known about such positional preference in rumen bacterial lipids, the *anteiso*-FA might be inserted in the *sn*-2 position to prevent excessive rigidity when SFA are increasingly inserted into the *sn*-1 position in rumen bacterial membranes. Vlaeminck et al. (2006b) reported a higher proportion of *anteiso*-FA compared with *iso*-FA in bacteria from cows fed high-concentrate diets. Some amylolytic bacteria are highly enriched in *anteiso*-FA and contain low *iso*-FA (Ifkovits and Ragheb, 1968; Miyagawa, 1982; Minato et al., 1988). More work is needed to compare differences in BCFA profile in amylolytic and cellulolytic bacteria as mediated by shifts in their environmental pH.

The *anteiso*-14:0 and *anteiso*-16:0 are unlikely to be derived from 2-methylbutyrate as would be the odd-chain *anteiso*-FA (Parsons and Rock, 2013). Further identification beyond our capacity was recommended before identifying even-chain *anteiso*-FA (Kaneda, 1991); hence, these FA were not tallied in the total *anteiso*-FA. Although α-oxidation could have reduced the chain length by 1 carbon, which is well recognized in aerobic cultures and has been shown in a few pure cultures of ruminal bacteria, its significance was deemed to be minor in the rumen (Vlaeminck et al., 2006a) and in batch culture of mixed rumen microbes (Wu and Palmquist, 1991). Ruminal bacteria are well known to make considerable amounts of fatty aldehydes (Stewart et al., 1997), many of which can be available as dimethylacetals after standard methylation procedures (Alves et al., 2013). The majority of those had chain lengths of 14 to 16 carbons (Ventto et al., 2017). Discerning the identity of these peaks potentially eluting at the same time as *anteiso*-14:0 and *anteiso*-16:0 standards (which could be produced in aerobic bacteria) would require commercially available dimethylacetal standards.

The main effect of k_p for odd-chain *iso*-FA (elongated from isovalerate) and interactions (or trends) of BCVFA with k_p for even-chain *iso*-FA (from isobutyrate), *anteiso*-FA, and total BCFA with increasing k_p support our expectation that faster growth of mixed bacteria requires more BCVFA incorporation into BCFA. A higher k_p could also have shifted bacterial populations toward those with faster potential growth, possibly explaining the trend for a modest increase in Simpson's diversity index (Table 3). Even though total bacterial N production was not affected by treatment (Roman-Garcia et al., 2021b), faster growth post-feeding could be equalized during the 12-h feeding interval, so these effects could be accentuated with multiple feeding events in vivo. Although we dosed racemic 2-methylbutyrate, the *S* enantiomer is probably preferred for incorporation into *anteiso*-FA in *Bacillus subtilis* (Kaneda, 1991) even though up to 5 to 10% could be derived from *R* enantiomers in ruminal bacteria (Eibler et al., 2017). Relative to isobutyrate and isovalerate, a benefit from exogenous 2-methylbutyrate is likely less reflected by a decreased ruminal concentration if the *R* enantiomers accumulate.

Our diet only had a moderate amount of dietary unsaturated fat. The decrease in *cis*-18:1 and *trans*-18:1 isomers with decreasing pH does suggest interruption of biohydrogenation at the lipolysis step, which would be supported by an approximate doubling of linoleic acid outflow in effluent from continuous culture (Qiu et al., 2004). Interactions of BCVFA with k_p (*cis*-18:1) and pH with k_p (*trans*-18:1) cannot be explained because our column could not consistently separate isomer peaks. Multiple *cis*-18:1 and *trans*-18:1 isomers result from biohydrogenation of linoleic and linolenic acid (Dewanckele et al., 2020).

Although valerate was assumed not needed and therefore not dosed in the BCVFA treatment (Roman-Garcia et al., 2021a), OCFA increased with decreasing pH (Table 1). Valerate production also increased (Roman-Garcia et al., 2021b), thus increasing competition with even-chain primers for FA synthesis. Both butyrate and valerate increased considerably with decreasing pH of continuous cultures (Berthelot et al., 2019). Valerate can be formed from degradation of some AA (Gorosito et al., 1985) but it is extensively formed from condensation of propionate with acetyl-CoA to consume [2H], much like valeryl-CoA is further elongated to OCFA (Ungerfeld, 2015b). The BCFA have higher melting points than the OCFA (Enser, 1984); when in lower pH environments that increase membrane fluidity, we would expect more 15:0 and less *iso*-FA to be incorporated into membranes (Fievez et al., 2012). Lower pH suppressed methanogenesis, which led to alternative [2H] sinks (Ungerfeld, 2015a), as supported by shifts in bacterial populations (see later discussion).

Relative Sequence Abundance of Prokaryotes

Diversity Indices. Continuous cultures had lower diversity but were still represented by major taxa from the original inoculum (Salfer et al., 2018). All 8 of the abundant core genera (Xue et al., 2018) were also abundant in our continuous cultures; in addition, heritable taxa (Wallace et al., 2019) were nearly completely represented, suggesting strong representation of ruminal conditions. The ASV clustering approach was chosen to improve accuracy and reduce technical errors associated with sequencing and computational analyses using operational taxonomic unit clustering approaches (Caruso et al., 2019). The average ASV that were observed (n = 1,308) or derived by the Chao 1 nonparametric statistic (n = 1,392) indicate a robust prokaryotic community. Although we noted no change in our study resulting from dosing BCVFA, when BCVFA were fed to lactating dairy cattle, the number of ASV and most indices of α-diversity increased (Lee et al., 2021). Our β-diversity results (Figure 1) support the major shifts in relative ASV abundance associated with pH, and the period effect reflects the important role of differing inocula sources. Our inocula was prepared from 4 lactating dairy cows (Roman-Garcia et al., 2021b).

Phylum Bacteroidetes. *Prevotella* and *BS11* were highly represented in the *Bacteroidetes*, which represented about half of all ASV in our study. The

Prevotellaceae are well known for the versatility of substrates used by its members (Deusch et al., 2017) and for metabolic efficiency, such as making ATP via electron transport phosphorylation (Deusch et al., 2019). *Prevotella* was strongly positively associated with subacute rumen acidosis, as was family *S24–7*, whereas family *BS11* was inversely associated (McCann et al., 2016). Even subclinical acidosis would promote periods of low feed intake and therefore slow ruminal k_p, explaining some of the interactions, such that *S24–7* might have outcompeted *BS11* with low pH and low k_p combinations. The uncultivated *BS11* likely is specific for a hemicellulose rather than starch (Solden et al., 2017), whereas *S24–7* is likely more amylolytic and opportunistic (Ormerod et al., 2016). Amylolytic bacteria should have more resilience to decreased ruminal pH. Little is known about *Bacteroidales* family *RF16*, but increasing dietary hay inclusion increased its relative abundance (Klevenhusen et al., 2017).

Phylum Firmicutes. Many shifts were observed within the members of the *Firmicutes* resulting from decreasing pH (Table 4). Both *Butyrivibrio* and *Pseudobutyrivibrio* increased in relative sequence abundance with increasing k_p, perhaps resulting from their unique ways to make ATP (Hackmann and Firkins, 2015). However, *Pseudobutyrivibrio* decreased with decreasing pH. The butyrivibrios have been grouped with respect to their ability to produce lactate (Palevich et al., 2019). Although characterized *Pseudobutyrivibrio* can produce lactate, their fibrolytic niche seems to be more restrictive than that of *Butyrivibrio*. The butyrivibrios are recognized for biohydrogenation through the *trans*-11 18:1 pathway, and the *Butyrivibrio* genus includes stearate producers that are more inhibited by linoleic acid when lactate accumulates (Paillard et al., 2007).

Decreasing pH increased genera of the *Veillonellaceae* (including *Succiniclasticum*, *Dialister*, and *Mitsuokella*) and *Erysipelotrichaceae* (*Bulleidia*), many of which are lactate and succinate producers or consumers (Deusch et al., 2017). *Mitsuokella* is closely related to *Selenomonas*, and *Succiniclasticum* is noted for its main niche to convert succinate to propionate (Stewart et al., 1997). Inhibition of methanogenesis increased expression of genes in propionate production via succinate particularly attributed to increases in *Prevotella* and in *Selenomonas* or other *Veillonellaceae* in goats (Denman et al., 2015). Increased *Prevotella* and *Veillonellaceae* help explain the simultaneous 28% decreases in both methane production and acetate:propionate ratio when pH was decreased (Roman-Garcia et al., 2021b). Many of the *Veillonellaceae* were also correlated with a shift toward the *trans*-10 18:1 pathway (Dewanckele et al., 2018).

The family *Ruminococcaceae* is widely recognized for its cellulolytic representatives, *R. albus* and *R. flavefaciens*; however, other members of this family have diverse substrates (La Reau and Suen, 2018). Although the *Ruminococcaceae* are prevalent in the particulate fraction and typically associated with cellulose and hemicellulose degradation (Denman et al., 2018; Moraïs and Mizrahi, 2019), the ruminal *Ruminococcus bromii* seems to be specifically amylolytic and prevalent in ruminants fed high-grain diets (Mukhopadhya et al., 2018). In support, relative abundance of ASV assigned to *R. bromii* was increased ($P < 0.01$) from <0.01% to 0.57% with high versus low pH treatments (data not shown). Therefore, increased amylolytic *Ruminococcus* species at the expense of cellulolytic species probably explains the lack of response in the total genus, whereas *Oscillospira* (family *Ruminococcaceae*) and uncharacterized *Ruminococcaceae* were apparently inhibited by decreasing pH. *Oscillospira* can have unique morphologies and potentially diverse niches in the rumen, but it was particularly abundant with ruminants fed fresh forage diets (Mackie et al., 2003), which should have higher ruminal pH. Characterized *Ruminococcus* spp. have clear requirements for BCVFA (Stewart et al., 1997), but we detected no effects of supplementation in our study, perhaps because of a shift among uncharacterized ruminococci that might not have the same requirements or because *Fibrobacter* outcompeted the cellulolytic ruminococci for the cellulolysis niche.

Phyla Fibrobacteres and Spirochaetes. The relative abundance of *Fibrobacter* increased when BCVFA were dosed and helped prevent a decreased abundance when pH decreased (Table 5). Its most consistent response to individual BCVFA in the literature was to isobutyrate, although 2-methylbutyrate also might stimulate growth (Roman-Garcia et al., 2021a). *Fibrobacter* spp. are now known to be more diverse than once thought but are still primarily cellulolytic specialists (Neumann and Suen, 2018). *Fibrobacter* spp. also have a major role in depolymerizing hemicellulose and pectin to support other members in the consortium even though they ferment only end products from cellulolysis. Both phylum *Fibrobacteres* and genus *Treponema* are considered core taxa in dairy cattle (Xue et al., 2018). *Treponema* spp. can have a direct role in degradation of pectin and possibly hemicellulose but also indirect roles attributed to cross-feeding with cellulolytics (Moraïs and Mizrahi, 2019). As those authors explained, *Fibrobacter* has a unique and complicated system to degrade fiber, including extracellular protein complexes and membrane-bound vesicles that pit plant cell walls. Ruminal treponemes probably are chemoattracted to cellulose and interact closely with or help

push the nonmotile *Fibrobacter* into close contact with the plant cell wall, cross-feeding on the resultant oligomers or sugars (Stanton and Canale-Parola, 1980, Harwood and Canale-Parola, 1984). *Treponema bryantii* requires isobutyrate and 2-methylbutyrate (Stanton and Canale-Parola, 1980), whereas *Treponema saccharophilum* requires isobutyrate (Paster and Canale-Parola, 1985) for growth. Both of these treponemes produce formate, although *T. bryantii* also produces succinate. All ruminal strains of *Fibrobacter* can produce formate, but all strains mainly produce succinate, which is subsequently fermented primarily to propionate and therefore associated with decreased methanogenesis (Neumann and Suen, 2018), supporting our previous discussion. Similarly, positive associations of succinate-producing *Fibrobacter* and *Treponema* with succinate-consuming *Succiniclasticum* and negative relationships with H_2-producing *Ruminococcus* (Xie et al., 2018) support greater considerations of networks to suppress methanogenesis without disrupting fiber degradation.

The expectation for *Treponema* to enhance adherence and fiber degradation by other community members (Kudo et al., 1987), including *Fibrobacter succinogenes*, likely involves BCVFA availability. Two strains of *F. succinogenes* had a relatively high concentration of *anteiso* odd-chain FA or aldehydes some of which increased in their membranes when a BCVFA solution was provided (Saluzzi et al., 1993). Roles of *anteiso*-15:0 in the glycolipid component of the membrane were noted for *F. succinogenes* and the oral spirochete *Treponema denticola* (Vinogradov et al., 2001). Those 2 bacteria reportedly lack the lipopolysaccharide components that are typical for other gram-negative bacteria. Moreover, the FA composition in the membrane of the cellulolytic vesicles of *Fibrobacter* (Moraïs and Mizrahi, 2019) has not been addressed. Although pure cultures of *Fibrobacter* had adherence inhibited by low pH, induced acidosis did not decrease its relative abundance (McCann et al., 2016). The dietary RDP in that study was not provided; however, based on the dietary composition, RDP was probably more than adequate to provide BCVFA. Based on our BCVFA × pH interaction, more work is needed to assess whether adequate BCVFA aid *Fibrobacter*'s resilience to periodic low pH compared with other cellulolytics.

Other Phyla. The relative increase in *Proteobacteria* (gram-negative) cumulative ASV and many of ASV assigned as genera in that phylum was at the expense of the relative decrease in most *Firmicutes* (most are gram-positive with a few exceptions such as the *Negativicutes*). These results correspond with the relative increase in 3-hydroxy 14:0 (Table 1), which is an important component in the cell wall of gram-negative bacteria derived endogenously (Parsons and Rock, 2013) and noted in many rumen bacteria (Miyagawa, 1982). The source of the 2-hydroxy 14:0 is less clear. It has been noted in family *Burkholderiaceae* in class β-*Proteobacteria* (Sohlenkamp and Geiger, 2016); in our survey, *Comamonadaceae* (order *Burkholderiales*) almost disappeared when pH declined. The prevalence of 3-hydoxy 14:0 but not 2-hydroxy 14:0 was noted in isolates presumptively identified as *Succinivibrio* (Miyagawa, 1982), but occurrence of 2-hydroxy 14:0 has received little attention for ruminal bacteria to our knowledge. *Succinivibrionaceae* are recognized for their starch degradation and succinate production (Denman et al., 2018), so their ability to thrive in low pH is expected. *Succinivibrionaceae* abundance is inversely proportional to methanogenesis (Wallace et al., 2015). This increase further supports the expanded role of succinate as an intermediate to explain the decreased methanogenesis, as described previously. Increased abundance of *Desulfovibrio* was also noted in cattle that emitted high amounts of methane (Wallace et al., 2015). Because aqueous H_2 is captured in sulfate reduction, *Desulfovibrio* compete with hydrogenotrophic methanogens, and their increase with decreasing pH might have lessened potential differences in aqueous H_2 concentration (Roman-Garcia et al., 2021b).

Methanobrevibacter is typically the most abundant archaeal genus in the rumen (Martínez-Álvaro et al., 2020) and was the dominant genus in our survey. *Methanomicrobium mobile* (hydrogenotrophic, not meeting our abundance threshold) requires all 3 BCVFA but especially 2-methylbutyrate in the highest concentration, whereas *Methanobrevibacter ruminantium* requires only 2-methylbutyrate (Tanner and Wolfe, 1988). *Methanobrevibacter ruminantium* strain M1's requirement was predicted to result from a gene lacking for isoleucine synthesis (Leahy et al., 2010). Although we did not detect any effect of BCVFA supplementation on *Methanobrevibacter* relative abundance, dosing BCVFA did increase methanogenesis, which was attributed in large part to improved NDF degradability (Roman-Garcia et al., 2021b). The suppressed methanogenesis at low pH might be a direct result of uncoupling of ATP synthesis resulting from changing the proton gradient needed for H^+-transport-coupled ATP synthesis (Russell, 1998). Uncoupling of methanogenesis from ATP usage for growth has been proposed. Although it needs empirical testing (Wallace et al., 2017), energy spilling was projected for ruminal methanogens based on nonrumen archaea (Russell, 2007). In addition to uncoupling of methanogenesis from growth, a shifting of individual species within *Methanobrevibacter* could also explain the varying efficiency of methanogenesis per its relative abundance (Danielsson et al., 2017).

Some inconsistencies in our data remain unresolved by direct comparisons of treatment effects within individual genera. For example, *Ruminobacter* specifically uses starch as substrate, so its decreased relative ASV abundance with decreasing pH was unexpected but might be a result of displacement by other bacteria that have more versatile substrate accessibility (Klevenhusen et al., 2017), perhaps because of *Ruminobacter*'s cellular location of amylase (Stewart et al., 1997). Little is known about the minor phyla represented in our study. The *F16* genus in phylum *TM7* is enriched in the fluid versus particulate phase (Jewell et al., 2015), and liquid phase *Tenericutes* increased with increasing hay intake (Klevenhusen et al., 2017). Deusch et al. (2017) noted that genes from *Tenericutes* and *Verrucomicrobia* were less and more abundant, respectively, relative to their abundance of operational taxonomic units. Further technical advances should help sort out more complicated relationships that were beyond the scope of our study.

CONCLUSIONS

Numerous genera from numerous phyla were shifted in response to changing pH, so effects of forage:concentrate need to be evaluated within the context of pH to decrease negative associative effects in dairy nutrition. 2-Methylbutyrate relative to the sum of isovalerate and especially isobutyrate appears to balance membrane fluidity resulting from fluctuations in pH by shifting *anteiso*-FA and *iso*-FA in bacteria. Interactions with pH and k_p suggest that BCVFA are more beneficial to help BCVFA-requiring cellulolytics and periodic decreases in pH resulting from increased intakes (and faster k_p) of mixed forage-grain diets by high-producing cows. Further work is needed to deconvolute ^{13}C enrichments of BCFA and BCAA to further expand on why ^{13}C was recovered in straight-chain FA and on conditions in which BCVFA can stimulate cellulolytic bacteria such as *Fibrobacter* and the secondary bacteria such as *Treponema* that cross-feed on fiber degradation products to improve NDF digestibility and possibly microbial protein synthesis in high-producing cows.

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ORCIDS

Y. Roman-Garcia https://orcid.org/0000-0001-8181-2470
K. E. Mitchell https://orcid.org/0000-0002-9769-3960
C. Lee https://orcid.org/0000-0002-4522-1232
M. T. Socha https://orcid.org/0000-0002-0227-6591
T. Park https://orcid.org/0000-0002-4480-4524
B. A. Wenner https://orcid.org/0000-0002-2333-1297
J. L. Firkins https://orcid.org/0000-0003-3518-1940