Stimulation of Microbial Protein Synthesis by Branched-Chain Volatile Fatty Acids in Dual Flow Cultures Varying in Forage and Polyunsaturated Fatty Acid Concentrations

Dissertation

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Abstract

Branched-chain amino acids (BCAA; valine, isoleucine, and leucine) can be deaminated by many amylolytic bacteria to branched-chain volatile fatty acids (BCVFA, isobutyrate, 2-methylbutyrate, and isovalerate), which are growth factors for some cellulolytic bacteria. Many cellulolytic bacteria cannot uptake BCAA or decarboxylate them, thus depending on cross feeding for BCVFA precursors for carboxylation to BCAA or synthesis of branched-chain fatty acids (BCFA; *iso* even-chain, *iso* odd-chain, and *anteiso* odd-chain) and branched-chain aldehydes (BCALD) found in bacterial phospholipid and plasmalogens, respectively.

Supplemental BCVFA and valerate, a straight-chain volatile fatty acid (VFA) that is also a growth factor for some rumen bacteria, have been previously supplemented together in a combination commonly referred to as 'isoacids'. However, prior in vivo studies have either provided only one BCVFA individually or all BCVFA and valerate but not different combinations of isoacids. Our objective in the first study was to determine an optimal combination of isoacids. Sixty (28 primiparous and 32 multiparous) lactating Jersey cows (106 ± 54 days in milk) were blocked and randomly assigned to either a control (CON) treatment without any isoacids, 2-methylburtyate (MB, 12.3 mmol/kg DM), 2-methylbutyrate and isobutyrate (MB + IB, 7.7 and 12.6 mmol/kg DM of MB and IB), or

all 4 isoacids (ISO, 6.2, 7.3, 4.2, and 5.1 mmol/kg DM of MB, IB, isovalerate, and valerate, respectively). The CON diet was fed for 2-wk covariate period, then cattle were fed their assigned treatment for the 8-wk sampling period (n=15). Daily intake and milk yield were recorded and samples from 4 consecutive milkings a week were analyzed for milk components. The milk fatty acid profile was analyzed on wk 5 and wk 9. Treatment tended to interact with parity for both fat and protein concentrations. Primiparous cows did not differ by treatment, whereas multiparous cows supplemented with MB + IB tended to have greater protein concentration compared to CON and MB treatments and greater fat concentration compared to all other treatments in multiparous cows. Though milk yield and dry matter intake (DMI) did not change with treatment, there was an interaction with week for lactation efficiency (measured as milk energy/DMI). Supplementation of MB + IB tended to increase lactation efficiency compared with CON during first interval (wk 3 and 4) and third interval (wk 7 and 8). However, cows fed MB alone had the numerically lowest lactation efficiency. The differences were greater during the earlier weeks of the study and decreased as cows entered late lactation. The percentage of 15:0 *anteiso* FA of milk fat was highest with cows fed MB, was greater than CON or MB + IB, but did not differ from ISO. In our study MB + IB and ISO both improved feed efficiency and not at the expense of average daily can, but MB + IB appeared to be the optimal combination.

Further research was needed for evaluating how BCVFA benefits vary under differing dietary conditions. The next study was an incomplete block design with 8 continuous cultures used in 4 periods with treatments (n = 4) arranged as a 2 × 2 × 2 factorial. The factors were: high (HF, 67% forage) or low forage (LF, 33% forage), without

or with supplemental corn oil (CO; 3% dry matter), and without or with 2.15 mmol/d each of isovalerate, isobutyrate, and 2-methylbutyrate. From each BCVFA, 5 mg/d of ^{13}C was dosed into each vessel. The isonitrogenous diets consisted of 33: 67 alfalfa:orchardgrass pellet, which replaced a concentrate pellet that mainly consisted of ground corn, soybean meal, and soybean hulls. The pH of the vessels was managed to minimize pH differences by diet.

The effects of BCVFA supplementation on nutrient degradation, ruminal fermentation, and prokaryotic profile were determined. Supplementing BCVFA increased neutral detergent fiber (NDF) degradability by 7.6% and improved bacterial N synthesis by 6.6% for organic matter truly degraded and by 6.5% for truly degraded N. The prokaryotic profile shifted mainly by diet. The relative sequence abundance decreased with LF for *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and genus *Butyrivibrio*. Recovery of the ^{13}C dosed as BCVFA decreased by 31% with LF relative to HF. Even though more label was recovered with HF, both NDF degradability and efficiency of bacterial N synthesis improved under all dietary conditions. Therefore, BCVFA supplementation could improve feed efficiency in dairy cows under diverse dietary conditions, even those that can inhibit cellulolytic bacteria.

To explore the effects of varied diets and BCVFA supplementation on bacterial lipid metabolism, total fatty acid (FA), bacterial FA, and bacterial aldehydes (ALD) flow from the vessels were collected and analyzed. The ALD are recovered after vinyl ether lipids are hydrolyzed from plasmalogen phospholipids. Supplementation of BCVFA did not influence biohydrogenation index but increased total FA flow leaving the vessels by

13.5%. The percent of BCFA in the bacterial FA profile decreased from 9.46% with HF to 7.06% with LF, and the percent of BCALD in the bacterial ALD profile decreased from 55.4% with HF to 51.4% with LF. Supplemental CO specifically decreased *iso* even-chain FA and *iso* even-chain ALD, indicating isobutyrate incorporation decreased in bacterial lipids. Dose recovery in bacterial lipids, BCFA, and BCALD decreased by 42.2%, 48.3%, and 29.0%, respectively, with LF versus HF. There was no appreciable label outside of branched-chain lipids. Although ALD were less than 6% of total bacterial lipids, they accounted for 26.0% of ^{13}C recovered in lipids. The recovery of the label in *iso* odd-chain FA was greater than *iso* even-chain FA, whereas recovery of *iso* even-chain ALD was greater than recovery in *iso* odd-chain ALD. However, most of the label was recovered with *anteiso* odd-chain FA and *anteiso* odd-chain ALD, indicating that 2-methylbutyrate was the BCVFA primer most used for branched-chain lipid synthesis. Both BCFA and BCALD are important for function and growth because membrane homeostasis is necessary to adapt membrane fluidity under different conditions. Therefore, BCVFA supplementation can provide the primers necessary for bacterial structure and can support the rumen microbial consortium.

Finally, the influence of dietary conditions and BCVFA supplementation on bacterial protein metabolism was explored. Lower forage in the diet increased bacterial BCAA flow by 9.12% and BCAA percentage in bacterial AA profile by 1.99%. Supplementation of BCVFA increased total AA flow by 13.0% and bacterial BCAA flow by 10.7%. Recovery of ^{13}C in bacterial AA C was higher with HF 96.3 µg/ mg compared to 66.1 µg/ mg with LF, despite greater bacterial BCAA flow with LF. The greater outflow

of total AA and bacterial BCAA would have post-ruminal effects, which would potentially explain prior post-absorptive responses from BCVFA supplementation to dairy cattle. The recovery was greatest with leucine, followed by isoleucine, and finally valine. Although isotope recovery in bacteria was greater with HF, BCVFA supplementation increased total AA and bacterial BCAA flow under all dietary conditions. Both increases could potentially explain post-absorptive responses from BCVFA supplementation.

In conclusion, 2-methylbutyrate is the BCVFA most generally needed by bacteria, supporting previous research in our lab. Isovalerate is generally used for leucine synthesis but much less so for lipids, also supporting our previous research showing it the least likely BCVFA to benefit bacteria. Although isobutyrate had the least carboxylation to its parent BCAA (valine), it seems to have a major role in synthesis of iso even-chain FA that are converted to vinyl ethers in plasmalogens. Further research is needed to understand this role, but the results do support why its combination with 2-methylbutyrate provided the best response in the lactation study.

Dedication

To my parents, Kristine and Dr. Kenneth Mitchell, who raised me with a love of the dairy industry and knowledge. They demonstrated every day what work ethic truly was and lead by example

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I want to thank all my family members who supported me during this time. My grandmother Karen Milke, who was always available to talk. My grandfather Raymond Milke, who passed in March 2021, was always a steadfast supporter of me and ended nearly every one of our conversations with, “if you do something you love you never work a day in your life”. To my grandparents Jeanette and Gerald Mitchell whom I credit my love of animals. Between my grandmother identifying local fauna to the grandchildren in her backyard or my grandfather asking if I could see the different personalities of cattle at work, it was easy to develop an appreciation of all animals. To my siblings Kathryn and Keith Mitchell, who remain the most self-motivated individuals I have met and continued to inspire me to push myself more. Then finally to Eduan Martinez Soto, my steadfast significant other who supported me during my time here. He was always completely supportive of my work but made sure I took breaks even when I refused to take them.

Thank you to my committee Dr. Zhongtang Yu, Dr. Chanhee Lee, and Dr. Robin White who gave me the time and training to let me develop into the researcher I am today. To my advisor and friend Dr. Jeffrey Firkins, you made me a better person, researcher, and teacher during my time at the Ohio State University. The impact of your mentorship on my life cannot be explained in words. The years working with you were the best years of my life. I wish it could last even longer and while I move into the next part of my career I am happy to have your continued support and friendship.

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Wenner, B. A., Park, T., **Mitchell, K.E.**, Kvidera, S. K., Griswold, K. E., Horst, E. A., and Baumgard, L. H. (2022). Effect of zinc source (zinc sulfate or zinc hydroxychloride) on relative abundance of fecal Treponema spp. in lactating dairy cows. JDS Communications. 3(5), 334-338.

Roman-Garcia, Y., **Mitchell, K. E**., Lee, C., Socha, M. T., Park, T., Wenner, B. A., and Firkins, J. L. (2021). 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. J. Dairy Sci., 104(9), 9868-9885.

Roman-Garcia, Y., **Mitchell, K. E.**, Denton, B. L., Lee, C., Socha, M. T., Wenner, B. A., and Firkins, J. L. (2021). 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. J. Dairy Sci., 104(9), 9853-9867.

Roman-Garcia, Y., Denton, B. L., **Mitchell, K. E.**, Lee, C., Socha, M. T., and Firkins, J. L. (2021). Conditions stimulating neutral detergent fiber degradation by dosing branched-chain volatile fatty acids. I: Comparison with branched-chain amino acids and forage source in ruminal batch cultures. J. Dairy Sci., 104(6), 6739-6755.

Lee, C., Copelin, J. E., Park, T., **Mitchell, K. E.,** Firkins, J. L., Socha, M. T., and Luchini, D. (2021). Effects of diet fermentability and supplementation of 2-hydroxy-4-(methylthio)-butanoic acid and isoacids on milk fat depression: 2. Ruminal fermentation, fatty acid, and bacterial community structure. J. Dairy Sci., 104(2), 1604-1619.

Rossow, H. A., Martinez, M. J. G., and **Mitchell, K. E**. (2020). Performance of preweaned Holstein calves given 2 phytogenic feed supplements in their starter grain. Appl. Anim. Behav., 36(6), 877-884.

Mitchell, K. E., and Rossow, H. A. (2020). Effects of a Glucose Precursor Supplement Fed to Holstein and Jersey Cows during the Transition Period on Ketosis Prevalence and Milk Production. EC Vet. Sci., 5, 1-13.

Fields of Study

Major Field: Nutrition

Table of Contents

Abstract ii

Dedication vii

Acknowledgments viii

Vita x

List of Tables xvii

List of Figures xxi

Chapter 1. Introduction 1

Chapter 2. Literature Review 7

Microbial Membrane Structure and Membrane Homeostasis 7

Dietary Fatty Acids in the Rumen and Biohydrogenation 13

Branched-chain Volatile Fatty Acids Production 19

Branched-chain Volatile Fatty Acid as Precursors of Branched-chain Amino Acids 24

Incorporation of Branched-chain Volatile Fatty Acids into Bacterial Branched-Chain Lipids 30

Odd Chain Fatty Acids and Branched-chain Long Chain Fatty Acids Incorporation in Milk Fatty Acids 34

Branched-chain Amino Acids In Vitro and In Vivo Experiments 36

Branched-chain Volatile Fatty Acids In Vitro and In Vivo Experiments 38

Chapter 3. Assessing milk response to different combinations of valerate and branched chain volatile fatty acids in primiparous and multiparous Jersey cows 54

Introduction....54
Material and Methods....56
Experimental design and treatments....56
Sample Collection, Processing, and Analyses....58
Statistical Analysis....60
Results....63
Body Weight and Condition Score....63
Milk Production and Milk Components....64
Intake and Efficiency....64
Fatty Acid Profile....69
Discussion....72
Conclusion....76
Chapter 4. Effects of branched chain volatile fatty acids supplementation on nutrient degradation, microbial functions, and prokaryotic profile in dual flow cultures varying forage and corn oil concentration....77
Introduction....77
Material and Methods....80
Experimental Design and Treatments....80
Continuous Culture Operation....81
Sample Collection and Processing....83
Statistical Analysis....86
Results....88
pH and Reduction/Oxidation Potential....88

Nutrient Degradation and Flow of Nitrogen 91
Net production of Volatile Fatty Acids, Methane Emission, and Aqueous Hydrogen 94
Isotope Recovery 95
Prokaryotic Profile Changes 98
Discussion 105
pH and Reduction/Oxidation Potential 105
Nutrient Degradation 106
Bacterial Protein Synthesis 108
VFA production, Gas production, and Isotope Recovery 109
Conclusion 111
Chapter 5. Effects of branched chain volatile fatty acids supplementation on biohydrogenation and incorporation into microbial lipids in dual flow cultures varying forage and corn oil concentration 113
Introduction 113
Material and Methods 116
Experimental Design and Treatments 116
Sample Collection and Processing 117
Separation of FAME and DMA 118
Gas Chromatography and Isotope Ratio Mass Spectrometry Procedure 120
Statistical Analysis 125
Results 126
Biohydrogenation Intermediates and Extent 126

Bacterial Fatty Acid Concentration and Profile 129
Bacterial Aldehyde Concentration and Profile 134
Isotope Recovery in Bacterial Lipids 139
Discussion 141
Biohydrogenation 141
Bacterial Lipids 143
Isotope Recovery 147
Conclusion 148
Chapter 6. Effects of branched chain volatile fatty acids supplementation on protein metabolism and incorporation into microbial protein in dual flow cultures varying forage and corn oil concentration 150
Introduction 150
Material and Methods 152
Experimental Design and Sample Collection 152
Amino Acid Analysis and Computations 153
Statistical Analysis 156
Results 156
Bacterial AA flow and profile 156
Isotope recovery in bacterial AA 160
Total AA flow and profile 162
Nonbacterial AA flow 163
Discussion 166
BCVFA usage by bacteria 166

AA supply 168
Conclusion 170
Chapter 7. Conclusions 171
References 175
Appendix A. Complete milk fatty acid profile 199
Appendix B. Aqueous hydrogen and gas production measurements of time point samples taken from dual flow continuous cultures 201
Appendix C. Volatile fatty acids concentrations of time point samples taken from dual flow continuous cultures. 204
Appendix D. Molar proportions of time point samples taken from dual flow continuous cultures 210
Appendix E. Completed bacterial fatty acids and fatty aldehyde flows and isotope recovery tables from dual flow continuous cultures 215
Appendix F. Completed amino acid flow and profile tables from dual flow continuous cultures 224

List of Tables

Table 3.1. Ingredient and nutrient composition (with standard deviations) of diets containing different combinations of branched-chain volatile fatty acids 62

Table 3.2 Lactation performance of Jersey cows fed different combinations of branched-chain volatile fatty acids 66

Table 3.3. Fatty acid profile of selected fatty acids and partial sums of milk produced of Jersey cows fed different combinations of branched-chain volatile fatty acids 71

Table 4.1 Ingredients and nutrients of the diets varying in forage and corn oil supplementation. 88

Table 4.2. Nutrient degradability, flow rates, and bacterial efficiency in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation. 93

Table 4.3. Net production of VFA, gas production, and dose recovery in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation. 97

Table 4.4. Alpha diversity metrics and prokaryotic profile in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation. 101

Table 5.1 Fatty acid composition of the diets varying in forage and corn oil supplementation. 117

Table 5.2 Effluent fatty acid flows and biohydrogenation of PUFA in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation. 128

Table 5.3. Bacterial fatty acid (FA) content, flow rate, and FA profile in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation. 131

Table 5.4. Aldehyde (ALD) composition from bacteria harvested from continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation. 137

Table 5.5. Dose recovery in bacterial lipids in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation. 140

Table 6.1 Amino acid composition of the diets varying in forage and corn oil supplementation. 154

Table 6.2. Bacterial AA concentration, efficiency of bacterial amino acid N (AA-N) synthesis, AA flow, and AA profile in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation. 158

Table 6.3. Dose recovery in bacterial AA in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation. 161

Table 6.4. Flow of nonbacterial AA in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation. 165

Table A.1. Complete fatty acid profile of milk produced by Jersey cows fed different combinations of branched-chain volatile fatty acids 199

Table B.1. Concentration aqueous hydrogen [H_2(aq)], H_2 and CH_4 production rate in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation..... 201

Table C.1. Concentration of VFA from in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation. 204

Table D.1. Molar proportions of VFA from in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation. 210

Table E.1. Bacterial fatty acids (FA) flows in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation. 215

Table E.2. Bacterial fatty aldehyde (ALD) flows in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation. 218

Table E.3. Dose recovery in bacterial fatty acids (FA) in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation. 220

Table E.4. Recovered dose distribution in bacterial fatty acids (FA) in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation. 221

Table E.5. Dose recovery in bacterial aldehydes (ALD) in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation. 222

Table E.6. Recovered dose distribution in bacterial aldehydes (ALD) in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation. 223

Table F.1. Bacterial AA flows in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation. 224

Table F.2. Effluent AA flows in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation. 225

Table F.3. Non-bacterial AA profile in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation. 226

List of Figures

Figure 2.1 Structure of a phospholipid and a plasmalogen showing the ester versus ether bond in the sn-1 location. 10

Figure 2.2 Example of dietary fats in ruminant diets..... 15

Figure 2.3 General pathway of biohydrogenation of linoleic, linolenic, and oleic fatty acids. 19

Figure 2.4 Production of branched-chain volatile fatty from branched-chain amino acids. 23

Figure 2.5 The general pathway for synthesis of branched-chain amino acids (BCAA). 26

Figure 2.6 Pentadecanoic acid is shown as an example of a straight odd chain fatty acid (A), an iso fatty acid (B), and an anteiso fatty acid (C). 31

Figure 2.7 Synthesis of branched-chain fatty acids (BCFA) from branched-chain volatile fatty acids (BCVFA). 33

Figure 3.1. Body condition score (BCS) of multiparous (panel a) and primiparous (panel b) Jersey cows fed either control (CON, no supplement), 2-methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO). 67

Figure 3.2. Average daily gain (ADG) of multiparous (panel a) and primiparous (panel b) Jersey cows fed either control (CON, no supplement), 2-methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO). 67

Figure 3.3. Milk component concentrations and SCC of multiparous (panel a) and primiparous (panel b) Jersey cows fed either control (CON, no supplement), 2-

methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO). 68

Figure 3.4. Milk nitrogen efficiency [milk N (kg/d)/DMI (kg/d)] for biweekly means from Jersey cows fed either control (CON, no supplement), 2-methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO). 68

Figure 3.5. Milk energy efficiency [NE_L (Mcal/d)/DMI (kg/d)] for biweekly means from Jersey cows fed either control (CON, no supplement), 2-methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO). 69

Figure 3.6. Anteiso fatty acid profile (g/100 g) in milk of multiparous (panel a) and primiparous (panel b) Jersey cows fed either control (CON, no supplement), 2-methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO). 72

Figure 4.1. Fermenter pH (panel a) and oxidation/reduction potential (ORP, panel b) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA). 90

Figure 4.2 Nonmetric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity of 16S rRNA gene relative abundance (rarefied data) from continuous cultures that were fed high (HF) or low forage (LF) diets ($P < 0.01$, top left), supplemented without corn oil (– CO, top right) or with corn oil (+ CO, $P = 0.40$), and without branched-chain VFA (–BCVFA, middle left) or with BCVFA (+ BCVFA, $P = 0.98$). The effect of study period (1-4) was $P < 0.01$ (middle right), but Fermenter (bottom) did not affect ($P = 0.96$)

the Bray-Curtis dissimilarity. There were no interactions (P > 0.97) among treatments. 104

Figure 5.1. Example thin layer chromatography plate used in the separation of fatty acid methyl esters (FAME, red rectangle) and dimethylacetals (DMA, black rectangle)..... 119

Figure 5.2. Flow chart describing the procedure for methylation of fatty acids (FA) and vinyl ether in plasmalogens and the separation of fatty acid methyl esters (FAME, red boxes) and dimethylacetals (DMA, black solid boxes). 123

Figure 5.3 Structure of phospholipid (a) and plasmalogen (d) during methylation procedure to free fatty acid (b) and aldehyde (e) before methylation to fatty acid methyl ester (FAME, c) and dimethylacetals (DMA, f). 125

Figure 6.1. Relative ratio of effluent AA flows (mg/d) of continuous cultures with branched-chain VFA supplementation/effluent flow of AA from continuous cultures without BCVFA supplementation. Bars marked with * indicate P ≤ 0.01 difference between the main effect means of with vs without BCVFA on AA flow. 163

Figure B.1 Fermenter aqueous H_2 [H_2(aq)] concentration over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA)..... 202

Figure B.2 Fermenter gaseous H_2 (top) and CH_4 (bottom) production rates over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA). Data are means from all 4 experimental periods taken on d 8 to 11 of the period. 203

Figure C.1 Concentration of total VFA (top) and acetate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA)..... 205
Figure C.2 Concentration of acetate (top) propionate (middle) and acetate: propionate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA)..... 206
Figure C.3 Concentration of butyrate (top), valerate (middle), and caproate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA). 207
Figure C.4 Concentration of isobutyrate (top) and 2-methylbutyrate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA). 208
Figure C.5 Concentration of isovalerate (top) and total branched-chain VFA (BCVFA, bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental BCVFA (– BCVFA) or supplemental BCVFA (+ BCVFA). ... 209

Figure D.1 Molar proportion of acetate (top) and propionate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA)..... 211

Figure D.2 Molar proportion of butyrate (top), valerate (middle), and caproate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA). 212

Figure D.3 Molar proportion of isobutyrate (top) and 2-methylbutyrate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA). 213

Figure D.4 Molar proportion of isovalerate (top) and total branched-chain VFA (BCVFA, bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental BCVFA (– BCVFA) or supplemental BCVFA (+ BCVFA). ... 214

Chapter 1. Introduction

The rumen microbiota is made up of numerous species of bacteria, protozoa, archaea, and fungi that can be generally grouped by the substrates degraded. These different groups produce various fermentation products as a result, but many of them also depend on using other groups' products—typically referred to as cross feeding—to fulfill their requirements for optimal function. Thus, these end-products of one group serve as growth-promoting factors for other groups. Depending on the diet and environment of the rumen, certain growth promoters can be limiting for optimal rumen microbial function and animal productivity.

A group of potential growth promotors are formed by degradation of branched-chain amino acids (BCAA; Val, Ile, Leu) by proteolytic bacteria and protozoa (contribution by fungi is not well characterized). The BCAA can be transported (perhaps as peptides) and preferentially incorporated into microbial protein or are deaminated and decarboxylated to branched-chain volatile fatty acid (BCVFA; isobutyrate, 2-methylbutyrate, isovalerate). Increasing the availability of BCVFA can improve microbial activity, especially the activity of some fiber-degrading microbes (e.g., Liu et al., 2014; Wang et al., 2015; Wang et al., 2018). Many characterized cellulolytic bacteria lack the complete enzymes to transport or synthesize their own BCAA from pyruvate (Allison,

1978; Allison et al., 1984). Therefore, they rely on a supply of BCVFA from different bacteria capable of deaminating and decarboxylating BCAA to BCVFA. Then, cellulolytic bacteria update BCVFA and elongate these primers into branched-chain lipids (BCL) for their microbial membrane or produce BCAA thorough reductive carboxylation (Allison et al., 1962a; Allison et al., 1962b; Allison and Bryant, 1963).

Previously, there was a product called IsoPlus™ (Eastman Chemicals, Kingsport, TN, USA), which was a mix of the 3 BCVFA and valerate. Valerate has commonly been included in these supplement mixtures because it was a growth factor for some strains of bacteria (Andries et al., 1987). The most consistent benefits of isoacid supplementation were either increased feed efficiency or increased milk production with similar DMI (Papas et al., 1984; Peirce-Sandner et al., 1985). However, due to handling issues and potential palatability concerns, the product was removed from the market (Rosener and Uhlenhopp, 1987). A new product called Isoferm The palatability concerns of IsoPlus™ were not distinguished from decreased voluntary DMI because in these previously mentioned studies, milk production was unchanged and resulted in improved milk efficiency. Current interest in improving production efficiency has encouraged revisiting isoacids and BCVFA supplementation for commercial herds.

Effective supplementation of isoacids or BCVFA requires determining factors that influence beneficial responses as well as exploring dosages and combinations of isoacids that optimize the usage of isoacids in commercial herds. Preliminary work with in vitro methods indicated that 2-methylbutyrate, which is formed by catabolism of Ile, was the BCVFA that was the most beneficial (Roman-Garcia et al., 2021a). However, the authors

commented that either isobutyrate or isovalerate in combination with 2-methylbutyrate increased neutral detergent fiber (NDF) degradability, whereas valerate supplementation provided no benefit. In a follow up study in continuous cultures, only BCVFA were supplemented, no valerate was provided to the cultures, and BCVFA supplementation increased NDF degradability (Roman-Garcia et al., 2021b).Prior to our work, there are no in vivo studies that explored different combinations of isoacids or BCVFA because previous studies have either explored different proportions of the 4 isoacids or supplemented only a single BCVFA or all 3 BCVFA (e.g., Papas et al., 1984; Liu et al., 2014; Wang et al., 2015; Wang et al., 2018).

Commonly reported responses from supplementation of BCVFA are increased NDF degradability and microbial N; however, not all studies report these benefits (Andries et al., 1987). For example, in a study with diet-induced milk fat depression, BCVFA and valerate supplementation alleviated milk fat depression but did not influence NDF degradability or N metabolism (Copelin et al., 2021). The milk fat depression was induced by decreasing forage in the diet and all diets contained safflower oil, which provided polyunsaturated fatty acids (PUFA). In contrast to BCVFA supplementation as a growth factor for cellulolytics, PUFA were bacteriostatic to cellulose-degrading and butyrate-producing bacteria (Maia et al., 2007; Maia et al., 2010). Supplementation of 2-methylbutyrate at different forage:concentrate diets improved NDF degradability, but supplementation was more beneficial with a higher inclusion of forage (Wang et al., 2018). When BCVFA were supplemented in continuous cultures, NDF degradability increased even under different pH and passage rate conditions (Roman-Garcia et al., 2021b).

However, despite increased abundance of key cross-feeding partners *Fibrobacter succinogenes* and *Treponema,* BCVFA supplementation did not influence efficiency of microbial protein synthesis (Roman-Garcia et al., 2021b; Roman-Garcia et al., 2021c). Therefore, the optimal BCVFA mixture may be diet-dependent.

As mentioned previously, BCVFA are primers for synthesis of BCL. In this dissertation BCL are referring to branched-chain fatty acids (BCFA) or branched-chain aldehydes (BCALD). The BCFA or BCALD can be categorized as *iso* even-chain lipids (derived from isobutyrate), *anteiso* odd-chain lipids (2-methylburyate), or *iso* odd-chain lipids (isovalerate). In order to maintain membrane homeostasis, many rumen anaerobic bacteria utilized BCL to adjust membrane fluidity to different conditions rather than unsaturated fatty acids because desaturation of fatty acids (FA) is typically requires O_2 (Kaneda, 1991; Zhang and Rock, 2008). In early single culture studies, ^{14}C from BCVFA was incorporated into both phospholipids and plasmalogens in bacterial membranes (Allison et al., 1962b). Plasmalogens are phospholipids from which an ester bond was reductively converted, in anaerobic bacteria, to an ether bond in the sn-1 position (Jackson et al., 2021). The role of plasmalogens is not defined, but in the membrane, plasmalogens pack more tightly, which can decrease membrane fluidity and permeability (Goldfine, 2017). However, plasmalogens could play a role in anaerobic bacteria by disrupting reactive oxygen damage (Jackson et al., 2021), although this role has not been verified in rumen bacteria. Because BCALD have been recovered in rumen bacteria (Alves et al., 2013), BCVFA primers for lipid synthesis can play an important role as branched lipids in plasmalogens in rumen bacteria.

Finally, BCVFA also serve as precursors for synthesis of BCAA. There are numerous cellulolytic bacteria that are characterized as incapable to transport or synthesize their own BCAA from pyruvate (Allison, 1978; Allison et al., 1984). Therefore, they rely on a supply of BCVFA primers to synthesize their own BCAA. This results in cross-feeding in which fibrolytic bacteria consume the BCVFA produced by other proteolytic bacteria (which are primarily amylolytic). Rumen bacteria that are incapable of BCAA synthesis without BCVFA primers have a low specific activity of isopropylmalate dehydrogenase and have limited ability to synthesize the isopropyl group necessary for BCAA production (Allison et al., 1966; Rogosa, 1971). Additionally, Allison et al. (1966) determined that the specific activity of isopropylmalate dehydrogenase in mixed rumen bacteria was overall low. This could indicate that rumen microbes may depend on BCVFA primers for BCAA synthesis to a greater extent than other microbial ecosystems. For fiber-degrading bacteria that have been characterized as unable to catabolize BCAA, supplementation of BCVFA could supply the carbon skeleton for BCAA synthesis for the rumen population.

A sufficient supply of BCVFA can support an efficient consortium in the rumen. However, different combinations and diet conditions likely influence BCVFA usage, so investigating these conditions can better determine how to apply supplementation in commercial settings. Therefore, the overall goals of this dissertation are to:

1) Investigate optimal combinations of BCVFA and valerate supplementation in multiparous and primiparous dairy cattle

2) Determine the influence of dietary conditions on responses to BCVFA supplementation in dual flow continuous cultures on…
    a. Nutrient degradation, efficiency of microbial protein synthesis, and prokaryotic profile
    b. Lipid metabolism and incorporation of BCVFA into microbial lipids
    c. Protein metabolism and incorporation of BCVFA into microbial protein

Chapter 2. Literature Review

Microbial Membrane Structure and Membrane Homeostasis

There is a wide variation in cell structure and membrane composition between rumen archaea, fungi, protozoa, and bacteria (Hobson and Stewart, 1988; Koga and Morii, 2005). Archaea consist of many unique polar lipids which generally consist of isoprenoid chains ether-linked to L-glycerol at the *sn*-2 and 3 positions, whereas bacteria and protozoa mainly consist of fatty acyl chains ester-bonded to D-glycerol (Langworthy, 1985; Koga and Morii, 2005). Ether bonds are also more resistant than ester bonds, which can account for some of archaeal resistance to extreme environments. A large portion of rumen fungi lipids are neutral fatty acids that consist mainly of diacyl glycerol, free fatty acids, triglycerides, and to a lesser degree tetrahymanol and squalene (Kemp et al., 1984; Body and Bauchop, 1985). The anaerobic fungi that have been studied did not have sphingolipids, glycolipids, plasmalogens, or phosphonyl lipids as a part of their membrane structure. Fungi also incorporate a large proportion of *cis* 18:1 fatty acid and saturated C12-18 fatty acids. Though sterols were found in *Neocallimastix frontalis* by Body and Bauchop (1985); however, in a reduced environment, steroid synthesis is unlikely, so tetrahymanol and squalene could serve to replace sterols in fungal membranes (Hobson and Stewart, 1988). Protozoal lipid membranes also differ from bacterial membranes due to the much

higher amount of unsaturated and conjugated linoleic fatty acids (CLA) (Viviani, 1970; Harfoot, 1978). Protozoa are capable of ingesting whole particles, including chloroplasts, which can be used in the lipid bilayer (Devillard et al., 2006; Huws et al., 2009). As a result, they incorporate a greater amount of unsaturated fatty acids in their membranes compared to bacteria. Moreover, protozoa (and fungi) are eukaryotes and have lipid-containing membranes encasing multiple organelles such as Golgi apparatus, endoplasmic reticula, digestive vacuoles and nuclei.

Bacteria can be generally grouped by their cell wall structure with the well-known Gram staining (Gram, 1884). Gram-negative bacteria have an inner and outer membrane with a thin peptidoglycan layer between these two membranes, whereas Gram-positive bacteria have a thick peptidoglycan layer outside of their single plasma membrane. The outer leaflet of the outer membrane of Gram-negative bacteria consists of lipopolysaccharides, protein, and phospholipids (Costerton, 1970). Some bacteria can cross-stain under different growth conditions, so 16S rRNA gene-based sequencing approaches have largely replaced gram-staining in identification.

Some rumen bacteria are also characterized as having a cell wall "coat" of carbohydrates external to the outer membrane in Gram-negative bacteria and the cell wall of Gram-positive bacteria. The "coat" was identified in *Fibrobacter succinogenes* (formally *Bacteroides succinogenes*), *Prevotella ruminicola* (formally *Bacteroides ruminicola*), and *Megasphaera elsdenii* (Costerton et al., 1974; Montgomery et al., 1988; Shah and Collins, 1990). That "coat" later was named the glycocalyx layer and has been identified and characterized outside the peptidoglycan layer of Gram-positive bacteria such

as *Ruminococcus flavefaciens* and *R. albus* (Latham et al., 1978; Costerton et al., 1981; Weimer et al., 2006). Similar structures can be found in archaea and protozoa as well, but rumen cellulolytic bacteria additionally depend on this layer for adhesion to substrates (Miron et al., 2001).

The high content of fatty aldehydes, which are found in both polar and nonpolar glycerol ether lipids, is a unique feature of rumen bacteria (Allison et al., 1962b; Wegner and Foster, 1963; Katz and Keeney, 1964). These aldehydes are found in plasmalogens, which are phospholipids with an ether linkage in the *sn*-1 position and are diagramed in Figure 2.1 (Goldfine, 2010a). These are unique to strictly anaerobic bacteria and are not found in aerobic or facultatively anaerobic bacteria, plants or fungi (Rezanka et al., 2012). Plasmalogens can be synthesized by higher organisms, but the anaerobic bacteria require a different pathway because of the lack of an oxygen-dependent desaturation mechanism that occurs in animal tissue (Goldfine, 2017). Anaerobic bacteria form plasmalogens by the reductive conversion of an ester bond in a phospholipid (Jackson et al., 2021).

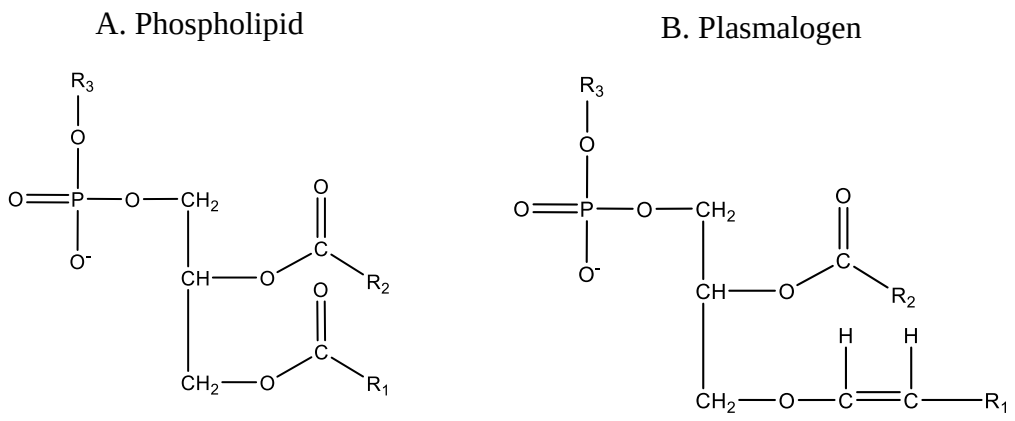

Figure 2.1 Structure of a phospholipid and a plasmalogen showing the ester versus ether bond in the sn-1 location.
Plasmalogens are formed anaerobically via a reductive step (Jackson et al., 2021) following phospholipid synthesis. The structures are similar except for an ester linkage in phospholipids versus an ether linkage in the plasmalogen structure. R_1 and R_2 represent acyl chain lipids (can be a branched-chain or straight chain lipid), while R_3 represents the head group of phospholipid structure (e.g. choline, serine, inositol).

Microbial survival depends on membrane homeostasis and the ability to adjust to different environments. These changes are mediated through biochemical and genetic mechanisms (Kaneda, 1991; Zhang and Rock, 2008). Studies investigating changing environments determined that alterations to the microbial membrane were to maintain fluidity to regulate passive permeability and membrane-bound enzymes. *Escherichia coli*, the model Gram-negative bacterium in gut microbiology, can maintain consistent fluidity with growth temperatures ranging from 15 to 43°C by increasing the ratio of saturated to unsaturated fatty acids in the membrane with increasing temperature (Sinensky, 1971; 1974). *Eubacterium cellulosolvens* 5494 (a Gram-positive member of the Clostridiales order) alters its membrane fatty acid profile to increase medium chain fatty acids and

unsaturated fatty acids, especially 18:1, to increase fluidity when cultured on a medium with cellulose compared to media with glucose, cellobiose, or fructose as energy sources (Moon and Anderson, 2001). These changes in profile were independent of pH or temperature. They suggested that this change in membrane composition is orchestrated with increased synthesis of the extracellular architecture needed to support cellulase activity. Even cellobiose only induced some of the cellulolytic enzyme components, but not the complete system (Blair and Anderson, 1999; Moon and Anderson, 2001). In the highly reduced rumen environment, desaturation of fatty acids for increasing membrane fluidity is virtually impossible because the currently characterized desaturases are oxygen-dependent (Aguilar et al., 1998; Zhu et al., 2006). However, dietary 18:1 fatty acids or intermediates of biohydrogenation have been incorporated into some rumen bacteria membranes (Hauser et al., 1979; Hazlewood and Dawson, 1979). Monounsaturated fatty acids (MUFA) and odd chain long chain fatty acids (OCFA) can be anaerobically synthesized from VFA, helping to maintain the fluidity of the microbial membrane (Fulco, 1983; Jenkins, 1993). Synthesis of MUFA from VFA has not been verified in rumen microbial populations and thus may not be a prominent adaptation for membrane homeostasis. Saturated fatty acids are more readily available due to biohydrogenation of polyunsaturated fatty acids (PUFA) in the rumen. Additionally, the bacteriostatic effects of PUFA on a large population of rumen microbes limit their use in microbial membranes.

Unlike protozoa, bacteria cannot ingest whole feed particles, so many depend on OCFA and branched-chain fatty acids (BCFA) to maintain membrane fluidity. The cellulolytic bacteria in the rumen contain a greater amount of *anteiso* odd, *iso* even, or *iso*

odd chain fatty acids (Vlaeminck et al., 2006). *Ruminococcus flavefaciens*, a predominant cellulolytic bacterium, is characterized by having a high proportion of *iso*-15:0 with some variation between strains. For example, *R. flavefaciens* FDI had mostly *iso*-15:0 compared to other OCFA or BCFA, whereas strain C94 still had a high proportion of *iso*-15:0, but also more *iso*-16:0 (Allison et al., 1962b; Ifkovits and Ragheb, 1968; Minato, 1988; Saluzzi et al., 1993). *Ruminococcus albus*, though in the same genus as *R. flavefaciens*, had a high proportion of *iso*-14:0 and a notable amount of *anteiso*-15:0, *iso*-16:0, and 15:0 (Ifkovits and Ragheb, 1968; Harfoot, 1978; Minato, 1988). Not all cellulolytic bacteria incorporate BCFA into their lipid bilayer at a high rate. *Fibrobacter succinogenes*, which is another major cellulolytic, does not include a high proportion of BCFA but, instead, maintains a greater proportion of 15:0 saturated fatty acids to maintain membrane fluidity (Ifkovits and Ragheb, 1968; Miyagawa, 1979; Minato, 1988; Saluzzi et al., 1993). Wegner and Foster (1963) noted that *F. succinogenes* S85 incorporated a major proportion of labeled isobutyrate into *iso*-14:0 and *iso*-16:0 acids. They also noted that ethanolamine plasmalogen was the major phospholipid identified for this strain. *Iso*-15:0 and *anteiso*-13:0 were the 2 major aldehydes identified with *F. succinogenes* ATCC 19169 (Minato, 1988). The role of plasmalogens and aldehydes in rumen bacteria is not defined and needs further exploration (Goldfine, 2017). As documented therein, plasmalogen lipids are more tightly packed than phospholipids, which decreases the permeability of membranes. Thus, BCFA precursors could play an important role in bacteria that incorporate plasmalogens. Unlike alkaline hydrolysis (Allison et al., 1962b), acid hydrolysis can release plasmalogen components. The fatty aldehydes can be converted to dimethylacetals (DMA) by methylation reactions and measured with the fatty acid methyl esters (FAME) that are also

formed (Oberg et al., 2012). These DMA can be important products in mixed ruminal bacteria as the chemical and physical structures of plasmalogens also provides plasticity to the bacteria membranes (Goldfine, 2010b). Additionally, the glycocalyx of *F. succinogenes* also contains anteiso-15:0 as glycolipids (Vinogradov et al., 2001), revealing another important role for branched-chain volatile fatty acid (BCVFA).

Although BCVFA is not essential to all the strains of studied cellulolytic bacteria for their membrane structure, they could still benefit from BCVFA supplementation as a source for carbon skeletons for branched-chain amino acid (BCAA) synthesis. Branched-chain fatty acids are not exclusive to cellulolytics. *Prevotella* spp. have a high proportion of *anteiso* C15:0 in their membrane (Miyagawa, 1979; Minato, 1988). Amylolytic bacterial membranes in general have low levels of BCFA, but a high amount of linear OCFA (Vlaeminck et al., 2006). Some species of amylolytic bacteria that follow this generalization include *Succinivibrio dextrinosolvens*, *Ruminobacter amylophilus*, and *Streptococcus bovis* (Ifkovits and Ragheb, 1968; Harfoot, 1978; Miyagawa, 1979; Minato, 1988).

Dietary Fatty Acids in the Rumen and Biohydrogenation

Dietary fats represent a small proportion of dry matter (DM) in ruminant diets but are commonly studied due to the unique interactions between the microbial population and lipids. High lipid content can decrease the overall digestibility of diets, especially fiber degradation. Protozoa and cellulolytic bacteria are more sensitive to increasing dietary lipids, especially those with higher proportions of PUFA (Hino and Nagatake, 1993). There

are a few theories on how this bacteriostatic effect occurs. One hypothesis is that the lipids can coat cellulolytic bacteria or feed particles; therefore, inhibiting their ability for the cellulosome structure to adhere to plant matter preventing normal cellulolysis (Jenkins, 1993). This theory is not well documented perhaps due to currently unfolding characterization of extracellular enzyme complexes using carbohydrate-active enzymes (Terry et al., 2019). Additionally, unsaturated fatty acids (UFA) may alter bacterial membrane fluidity, thereby changing permeability and inhibiting normal cell function. When there is no free carboxyl group on fatty acids (FA), these negative effects are alleviated (Jenkins and Palmquist, 1984). Hackmann and Firkins (2015a) hypothesized that differences in *iso* and *anteiso* fatty acid structure of cellular membranes might be associated with resilience against differential toxicity to UFA.

The fat in forage stems and leaves can represent up to 8% of dry matter; unsaturated fatty acids (especially 18:3) are localized as galactolipids in chloroplasts that are 22-25% lipid on a dry matter basis (Shorland, 1963; Harfoot, 1978; Jarrige et al., 1995). Most studies report that glycolipids are the main lipid class of forages. Glycolipids have FA in the *sn*-1 and *sn*-2 position of the glycerol backbone and a carbohydrate attached by a glycosidic bond in the *sn*-3 position. Monogalactosyldiglyceride (Figure 2.2, A) and digalactosyldiglyceride are the most common glycolipids, with either a single galactose molecule or 2 molecules in the *sn*-3 glycerol position (Roughan and Batt, 1969; Tremolieres, 1970). Dietary fat entering the rumen will also consist of phospholipids (Figure 2.2, C), which make up the chloroplast membrane structure (Shorland, 1963; Jarrige et al., 1995). The proportion of the different lipids varies under growth conditions.

For example, alfalfa grown at lower temperatures has a greater proportion of galactolipids; at higher growth temperatures, phospholipid concentration increases (Kuiper, 1970). The fatty acids found in concentrates, especially oilseeds, are stored as triglycerides (Figure 2.2, C). Forages and concentrates also differ in their fatty acid profiles. The major FA in forages are α-linolenic (*cis*-9, *cis*-12, *cis*-15-18:3) and linoleic (*cis*-9, *cis*-12-18:2) to a lesser degree (Garton, 1959; Kuiper, 1970). Concentrate lipids consist mainly of linoleic and a lower proportion of oleic FA (cis-9-18:1) (Viviani, 1970).

A. Monogalactosyldiaclyglycerol B. Phospholipid C. Triglyceride

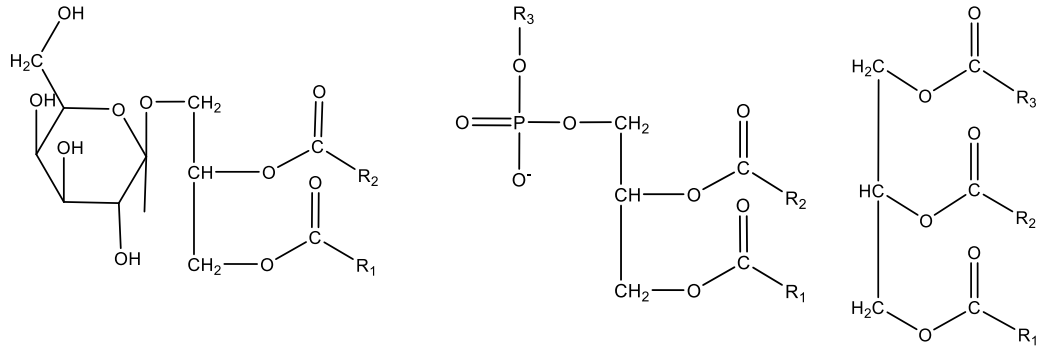

Figure 2.2 Example of dietary fats in ruminant diets.
Where R_x indicates a hydrocarbon tail where x indicates the sn location of a carbon chain. Except for R_3 in Figure B where this indicates an organic molecule such as choline, ethanolamine, serine, inositol, etc. bound to the phosphate group in the sn-3 position.

Rumen metabolism of lipids begins with the hydrolysis of the ester linkages. Diglycerides and monoglycerides are rapidly hydrolyzed, which releases the fatty acids after which the remaining structures can be fermented (Noble et al., 1974). Plant-based esterases and phosphatases were suspected to be responsible for the rapid hydrolysis of plant lipids, especially triglycerides (Dawson and Hemington, 1974; Faruque et al., 1974). In a study by Faruque et al. (1974), plant-based enzymes were still present in the soluble fraction of rumen fluid up to 5 hours after feeding. There was also very little lipolytic activity in rumen fluid from animals subjected to overnight fasting; thus, they concluded that plant-based enzymes played a major role in lipid metabolism. Dawson *et al.* (1977) destroyed ovine microbial enzymes with heat treatment, noting that ^{14}C-labeled galactolipids and triolein were not degraded when incubated under these conditions. Further research concluded that the lipases responsible for the degradation of plant lipids were of microbial origin because results that supported Faruque et al. (1974) were difficult to replicate (Garton et al., 1961; Dawson and Hemington, 1974; Dawson et al., 1977).

The earliest lipolytic bacterial isolate was named *Anaerovibrio lipolytica* strain 5S (Hobson and Mann, 1961; Hungate, 1966). This bacterium did not ferment common sugars and thus was placed into a new genus (Hungate, 1966). Triglycerides and especially diglycerides were hydrolyzed by the extracellular lipase enzyme from *A. lipolytica* (Henderson, 1971). Because phospholipids and galactolipids were not hydrolyzed by *A. lipolytica*, other lipolytic bacteria were isolated. *Butyrivibrio* species were assigned a major role in lipid metabolism, with lipase activity for dietary triglycerides, phospholipids, sulfolipids, and galactolipids (Latham et al., 1972; Hazlewood and Dawson, 1975;

Hazlewood and Dawson, 1979). Some early identified *Butyrivibrio* strains included *B. fibrisolvens* LM8/1B, which lacked cellulolytic activity but showed lipase activity for phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol (Hazlewood and Dawson, 1975). *Butyrivibrio* strain S2 was then isolated with only grass galactolipids as the carbon source in the medium. This long chain fatty acid (LCFA) auxotroph hydrolyzed ester linkages of galactolipids, phospholipids, sulpholipids, and diglycerides, then incorporated the essential LCFA into its membrane structure (Hazlewood and Dawson, 1977; 1979).

Early researchers also consistently noted that bacteria isolated for lipase activity of phosphatidylcholine also were capable of hydrogenating UFA (Hazlewood et al., 1976). Biohydrogenation was known to be unique to ruminants since Banks and Hilditch (1931) found that the FA profile of adipose tissue was more saturated in ruminants than in non-ruminants. The FA profile difference was later attributed to changes that occurred in the rumen and not in the adipose tissue as originally hypothesized by Banks and Hilditch (1931). Biohydrogenation serves to detoxify FA that are toxic to many of the bacteria in the rumen (Kemp and Lander, 1984). Isolation of bacteria capable of biohydrogenation remained an issue because of the diverse substrates utilized by the species. Researchers were forced to start with isolating pure cultures and then screen them for their ability to biohydrogenate PUFA, which was very tedious because bacteria must be actively growing (Polan et al., 1964; McKain et al., 2010).

Biohydrogenation occurs after lipolytic bacteria hydrolyze lipids and release the free UFA. A free carboxyl group is necessary for the microbial enzymes performing this

process (Kepler et al., 1970). The main pathway of biohydrogenation for α-linolenic fatty acids begins with isomerization to 18:3 *cis*-9, *trans*-11, *cis*-15, then hydrogenation of the *cis* double bonds to 18:2 *trans*-11, *cis*-15 and then 18:1 *trans*-11. The final step, which is slower, is the hydrogenation of the *trans* bond to stearic acid (Figure 2.3). Most of the linoleic acid is isomerized to *cis*-9, *trans*-11-CLA, the *cis* bond is hydrogenated to produce 18:1 *trans*-11, and the latter is finally hydrogenated to stearic acid (Ward et al., 1964; Noble et al., 1974). The multiple researchers who identified bacteria capable of biohydrogenation determined few bacteria completely hydrogenated PUFA to stearic acid (Hazlewood et al., 1976; Kemp and Lander, 1984). For example, *B. fibrisolvens* can isomerize and reduce linoleic and α-linolenic to 18:1 *trans*-11, but not completely to 18:0. *Fusocillus babrahamensis* P2/2 and *Fusocillus* T344 reduced the final trans bond (White et al., 1970; Kemp et al., 1975; Hazlewood et al., 1976). Although these isolates were lost, they were closely related to *Butyrivibrio proteoclasticus*, which is capable of complete biohydrogenation of linoleic acid (Wallace et al., 2006; Moon et al., 2008). The steps identified by these researchers are marked in Figure 2.3 by the bolded pathway, the major pathway for PUFA saturation. The 18:1 *trans*-11 intermediate is the most common and tended to accumulate due to the last reduction step being slower than the reduction of the *cis* bonds. An alternate pathway for biohydrogenation UFA, mainly linoleic fatty acids, is the isomerization to *trans*-10, *cis*-12-CLA, which is then reduced to 18:1 *trans*-10 is also included in Figure 2.3.

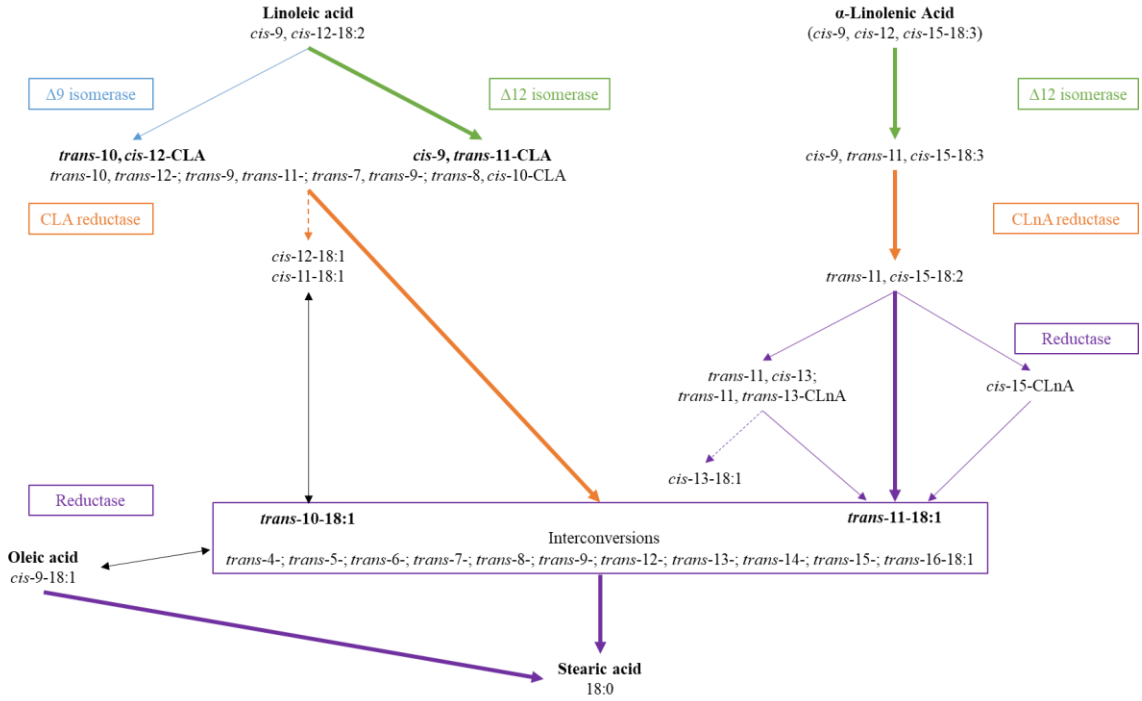

Figure 2.3 General pathway of biohydrogenation of linoleic, linolenic, and oleic fatty acids. General pathway of biohydrogenation of linoleic, linolenic, and oleic fatty acids. The bolded lines indicate the most common intermediates formed during biohydrogenation in mixed rumen microbes. Conjugated linoleic acid (CLA), conjugated linolenic acid (CLnA). Adapted from (Enjalbert et al., 2017)

Branched-chain Volatile Fatty Acids Production

The BCVFA are short chain fatty acids with a branched methyl group that are formed primarily by the deamination of BCAA by non-cellulolytic bacteria in ruminants. When BCAA were limited in media, *Prevotella ruminicola*, *P. brevis*, and *Megasphaera elsdenii* decreased production of BCVFA, demonstrating that BCAA are the source of BCVFA (Allison, 1978). Other bacteria studied do not produce BCVFA even when provided with BCAA; these groups include *Streptococcus bovis*, *Selenomonas*

ruminantium, *Ruminobacter* (formerly *Bacteroides*) *amylophilus*, and *Butyrivibrio fibrisolvens* (Allison, 1978; Stackebrandt and Hippe, 1986). The first step of BCAA (Leu, Ile, and Val) fermentation is the deamination or transamination by branched-chain aminotransferase (BCAT) to the corresponding α-ketoacids (α-ketoisocaproate, α-ketomethylvalerate, and α-ketoisovalerate) by BCAT in *Bacillus* spp. (Dehority et al., 1958; Kaneda, 1977; Annous et al., 1997). The α-ketoacids are rapidly dehydrogenated to the acyl-coenzyme A (CoA) derivatives (isovaleryl-CoA, 2-methylbutyryl-CoA, and isobutyryl-CoA) by branched-chain α-keto acid dehydrogenase. The remaining acyl-CoA derivatives can be converted to corresponding carboxylic acids by acyl-CoA hydrolase. Production of BCVFA can lead to cross-feeding when the resulting BCVFA are used by other populations of bacteria for synthesis of BCAA or BCFA (Miura et al., 1980; Firkins, 2010).

Early research on BCAA catabolism noted the production of ammonia and carbon dioxide as some of the byproducts (El-Shazly, 1952a; b). The dissimilation of BCAA by anaerobes includes a mechanism called the Stickland reaction (Nisman, 1954). The reaction couples an oxidation-reduction between pairs of amino acids. When DL-Val-1-^{14}C was added, it was decarboxylated and $^{14}CO_2$ contained most of the radioactivity (Dehority et al., 1958). In the same study, the *in vitro* incubation was decreased to 20 h (versus 30 h) and the temperature was lowered to 25°C (versus 39°C) to be able to measure α-ketoisovaleric acid. They confirmed that a deamination step occurred prior to the rapid decarboxylation of the substrates. Whereas D-Val was not metabolized. The initial steps of BCAA metabolism in this study followed a similar pathway in mammalian tissue, and

Dehority et al. (1958) theorized Leu and Ile would also follow similar pathways. Later, it was determined that BCAA were mainly transaminated by BCAT, and another α-ketoacid was an acceptor (Rudman and Meister, 1953; Massey et al., 1976). In *Escherichia coli*, α-ketoglutarate was an acceptor for BCAT (Rudman and Meister, 1953). In mixed rumen fluid, there is evidence for multiple transaminases, but BCAT utilizes α-ketoglutarate and BCAA for glutamic acid production in sheep rumen fluid (Ichihara and Koyama, 1966; Tsubota and Hoshino, 1969).

In the rumen, BCAA catabolism is directly linked to methanogenesis, which is diagrammed in Figure 2.4 (Hino and Russell, 1985). When monensin or carbon monoxide was added to decrease methane production, deamination decreased, especially that of the BCAA. The intracellular NADH: NAD was increased by hydrogenase inhibitors. The authors then continued the experiment by adding either NADH or methylene blue, which oxidized NADH. Additional NADH also decreased deamination of BCAA and formation of ammonia, whereas methylene blue stimulated deamination. Small additions of α-ketoglutarate also enhanced BCAA deamination, which indicates transamination by glutamate dehydrogenase was a factor in catabolism.

Branched-chain volatile fatty acids are not necessarily terminal end products of BCAA catabolism. Apajalahti et al. (2019) assessed BCVFA concentrations to estimate ruminal protein degradation and reported that their results might have been affected by BCAA incorporation into bacterial protein, utilization of acyl-CoA derivatives for BCFA synthesis, or further catabolic processes. The initial steps followed what was outlined previously by Proteobacteria, but the acyl-CoA derivatives of BCAA can further be

metabolized by individual catabolic pathways to propionyl-CoA or acetyl-CoA (Kazakov et al., 2009). In *Proteobacteria*, BCAT was identified along with an enzyme (Leu dehydrogenase) that acted on only Leu. In *Clostridioides difficile*, a reductive and oxidative Stickland pathway has been identified (Neumann-Schaal et al., 2019). The initial steps of amino acid catabolism are the same as before with BCAT with an α-ketoglutarate acceptor. The α-ketoacids then can enter either pathway, but BCVFA are produced by the oxidative Stickland reaction. In this pathway, acyl-CoA derivatives are produced from α-ketoacids by α-ketoacid oxidoreductase; CO_2 and 2 reduced ferredoxins are also produced. Adenosine triphosphate formation occurs when phosphate acyltransferase and carboxylic acid kinase (or CoA ligase) produce the carboxylic acids from acyl-CoA derivatives. The complete pathways for BCAA fermentation in rumen bacteria still are incompletely described by current research. With more mechanistic studies and genome sequencing, this literature will become more complete and help understand the complex rumen ecosystem.

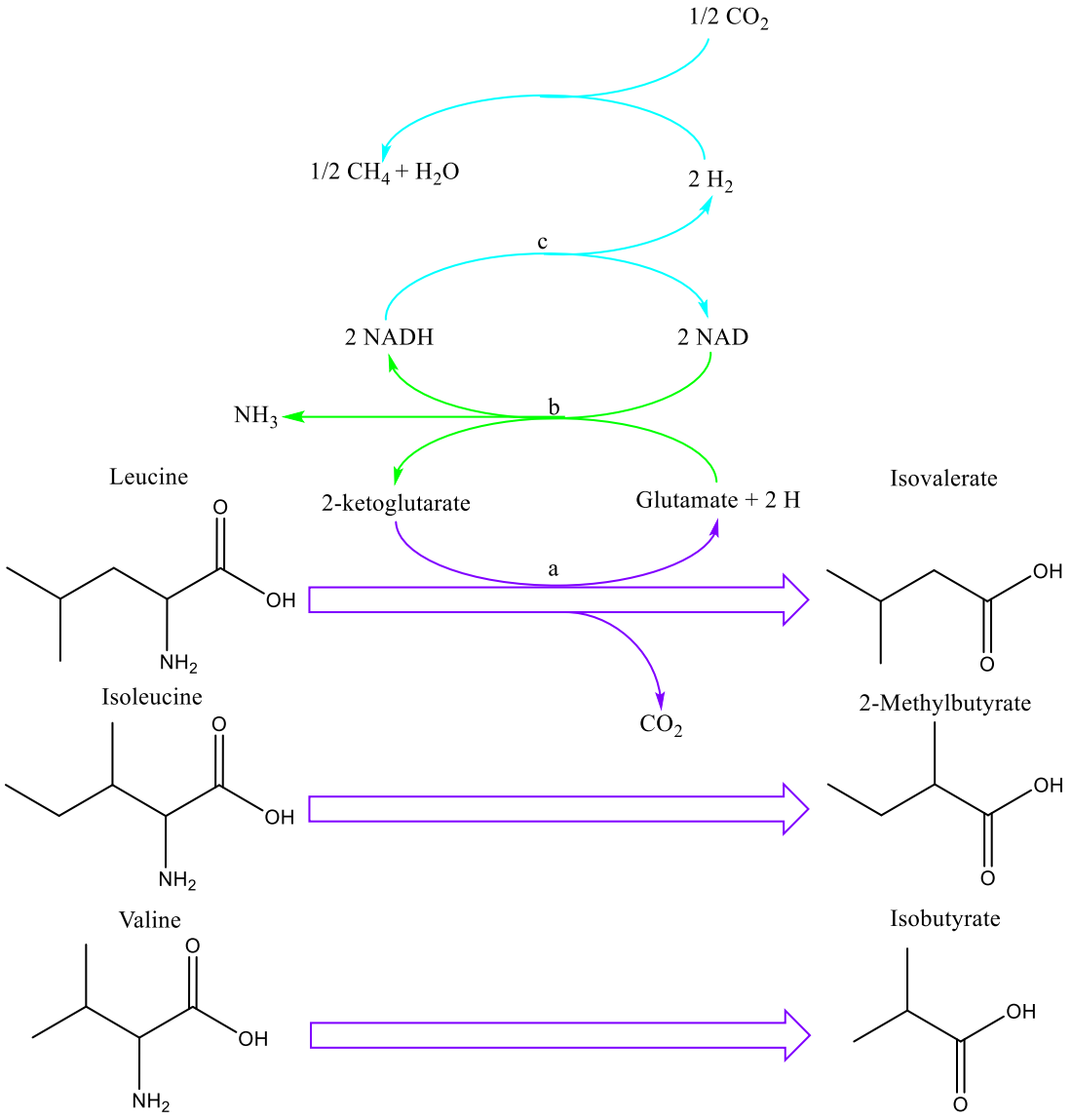

Figure 2.4 Production of branched-chain volatile fatty from branched-chain amino acids. Each BCVFA has a methyl group either on the second (isobutyrate and 2-methylbutyrate) or third (isovalerate) Δ carbon. Fermentation of BCAA to BCVFA occurs via transamination and decarboxylation (a, purple) and the disposal of the reducing-equivalents (b, green) by methanogenesis (c, blue) in mixed rumen microbes. Adapted from (Dehority et al., 1958; Hino and Russell, 1985).

Branched-chain Volatile Fatty Acid as Precursors of Branched-chain Amino Acids

Anaerobic bacteria can synthesize Val, Leu, and Ile by the BCAA synthesis pathway. Figure 2.5 diagrams a simplified pathway that includes the precursors, important intermediates, and final products. The first step is catalyzed by Thr dehydratase/deaminase, which converts Thr or Ser to α-ketobutyrate; this step experiences negative feedback from L-Ile in *E. coli* (Umbarger, 1956; Umbarger and Brown, 1958). The 2nd step is mediated by acetohydroxyacid synthase, which is universally shared between the 3 pathways to the 3 different BCVFA. The enzyme decarboxylates pyruvate and condenses with either 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate to begin synthesis of Ile or with pyruvate (Val pathway branch or right side of Figure 2.5) to 2-acetolactate (Gollop et al., 1989). The enzyme is inhibited by end product feedback with L-Ile, but more strongly with L-Val in *Mycobacterium tuberculosis* (Kim et al., 2005). Ketol-acid isomeroreductase then converts the products of acetohydroxyacid synthase to 2,3-dihydroxy-3-methylvalerate and 2,3-dryhydroxyisovalerate, respectively. This has been confirmed in *E. coli*, *Neurospora crassa*, and *Saccharomyces cerevisiae*; both conversions are performed by a single enzyme, though 2 classes have been identified (Radhakrishnan et al., 1960; Umbarger et al., 1960; Wixom et al., 1960; Amorim Franco and Blanchard, 2017). Dihydroxy acid dehydratase then synthesizes 2-keto-3-methylvalerate and 2-ketoisovalerate; this enzyme is also required for the synthesis of pantothenate, which is also shown in Figure 2.5 (Myers, 1961). The intermediate 2-ketoisovalerate is used for BCAA synthesis and pantothenate synthesis. The first committed step for L-Leu synthesis utilizes isopropylmalate synthase, which converts 2-ketoisovalerate with acetyl-CoA to 2-isopropylmalate and CoA, which

then is isomerized to 3-isopropylmalate by isopropylmalate isomerase and finally oxidized and decarboxylated by isopropylmalate dehydrogenase to 2-ketoisocaproate. The final step for BCAA synthesis is the transfer of the α-amino group from L-glutamate to the α-carbon of 2-ketoisovalerate (L-Val), 2-ketomethylvalerate (L-Ile), and 2-ketoisocarpoate (L-Leu) by BCAT, which is a reversible reaction in *M. tuberculosis* (Amorim Franco et al., 2017).

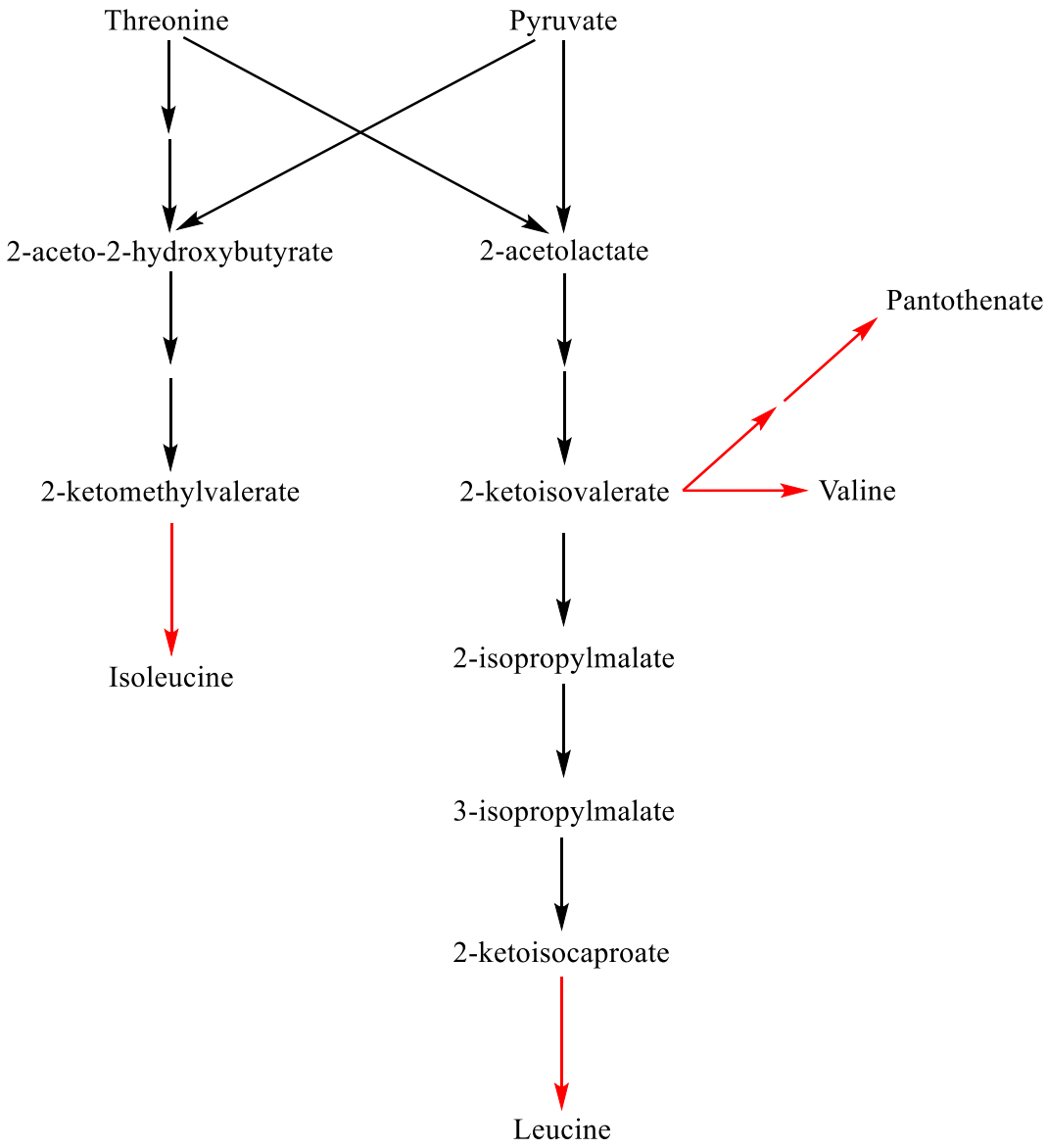

Figure 2.5 The general pathway for synthesis of branched-chain amino acids (BCAA). Pathway for synthesis of Ile, Val, and Leu that is common to many anerobic bacteria for synthesis of BCAA without BCVFA as the initial substrates. This pathway can also synthesize pantothenic acid (Vitamin B_5). Adapted from (Stieglitz and Calvo, 1974; Amorim Franco and Blanchard, 2017).

Pantothenic acid (PA) is mentioned due to its shared pathway to BCAA synthesis and its metabolic importance for incorporation into CoA and acyl carrier protein (ACP) among other roles (Stieglitz and Calvo, 1974; Ball, 2006; Amorim Franco and Blanchard, 2017). Similar to BCVFA, PA is required by some microbes of the rumen and therefore may have growth-promoting potential for different populations. Besides its use as a cofactor by rumen microbial metabolism, cattle depend on bacteria for synthesizing their required vitamin B supply. High-producing animals may need supplementation, so research has focused on the flow of vitamin B to the duodenum besides its rumen effects (NRC, 2001). Both *Streptococcus bovis* and *Megasphaera elsdenii* required PA for growth (Hungate, 1966). However, supplemental PA typically benefited rumen cellulolytic bacteria. Pantothenic acid increased acetate molar proportion *in vitro*, with very few other effects (Völker et al., 2011). When a partially rumen protected PA was supplemented in Blonde d'Aquitaine × Simmental calves, there were numerous notable benefits, which included increased DMI, average daily gain (ADG), nitrogen balance, and NDF digestibility (Liu et al., 2018d). In an experiment with dairy bulls supplemented with PA, acetate: propionate ratio, NDF total tract digestibility, xylanase, and pectinase activity increased (Wu et al., 2019). Additionally, Wu et al. (2019) used quantitative real-time polymerase chain reaction (qPCR) to target bacterial populations. Total bacteria, *Ruminococcus albus*, *R. flavefaciens*, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, and *Ruminobacter amylophilus* increased, but *Prevotella ruminicola* did not. Similar results were reported by Liu et al. (2017), but PA also increased *Prevotella ruminicola* relative abundance and activities of carboxymethylcellulase, cellobiase, protease, and α-amylase.

Microbes that express the pathway diagramed in Figure 2.5 do not have to rely on BCVFA or BCAA availability in the rumen to fulfill their requirements for BCAA, but pyruvate also competes with other pathways needed for cellular growth. There are key populations such as *Ruminococcus flavefaciens*, *Prevotella ruminicola,* and *Megasphaera elsdenii* (formally *Peptostreptococcus elsdenii*) that have a very low specific activity of isopropylmalate dehydrogenase compared to *E. coli* (Allison et al., 1966; Rogosa, 1971). These populations cannot depend on the isopropylmalate pathway and require BCVFA as a supply for BCAA synthesis. *Ruminococcus flavefaciens* strain C94 required isobutyrate or isovalerate for growth; with isovalerate-1-^{14}C dosed, all radioactivity in protein was in the 2nd carbon of Leu (Allison et al., 1962a). This same study showed that *R. flavefaciens* C94 had a very limited ability to use exogenous amino acids and DL-Leu and 2-ketoisocaproate could not replace the requirement for isovalerate. They also demonstrated that *R. flavefaciens* could not synthesize the isopropyl group necessary for BCAA production. Unlabeled isobutyrate, isovalerate, and 2-methylbutyrate were utilized as carbon skeletons for BCAA by *R. flavefaciens,* and the carbon for the carboxylation step came from radioactive CO_2, and not formate carbon or acetate-2-^{14}C (Allison and Bryant, 1963). The ability to incorporate BCVFA into BCAA has been noted in many major rumen microbial populations. *Prevotella ruminicola* strain 23 incorporated 2-methylbutyrate-1-^{14}C into Ile, and all of the radioactivity was in carbon 2 (Robinson and Allison, 1969). *Megasphaera elsdenii* also used this alternate pathway for BCAA production (Allison et al., 1966). In this same study, Allison et al. (1966) determined that the specific activity of isopropylmalate dehydrogenase in mixed rumen bacteria was low and suggested that the rumen microbes may depend more on BCVFA as carbon skeletons for BCAA

synthesis than other microbial ecosystems that researchers have studied. This supposition was supported by the lower than expected incorporation of acetate-2-^{14}C into Leu *in vitro* with sheep rumen contents. Based on these studies, synthesis of BCAA from BCVFA incorporates the intact BCVFA and CO_2 via a reduction carboxylation reaction (Allison et al., 1962a; Allison and Bryant, 1963; Robinson and Allison, 1969).

Although uptake of preformed BCAA is important in mixed ruminal microbes (Atasoglu et al., 2004), little is known about the expression of transporters among bacterial groups. Conversely, although BCVFA incorporation into BCAA has been documented with isotopes, few studies have verified the actual mechanism involved. Until more mechanistic information becomes available, isotope studies offer the best way to study further our understanding of BCAA or BCVFA usage in mixed microbes. Cellulolytic bacteria are a predominant group that may require BCVFA, but other microbes may also benefit. For example, *Selenomonas ruminantium*, *Ruminobacter amylophilus*, and *Butyrivibrio fibrisolvens* did not produce any BCVFA when BCAA were provided (Allison et al., 1962a; Robinson and Allison, 1969; Allison, 1978; Allison et al., 1984). This potentially means they do not express transporters or enzymes to catabolize BCAA, and thus may not have the substrates necessary to synthesize either BCFA or BCAA unless otherwise provided. Since these foundational studies, our understanding of the microbial diversity of the rumen microbiota has greatly expanded, especially of the *Bacteroidetes* phylum, which is poorly represented by characterized isolates.

Incorporation of Branched-chain Volatile Fatty Acids into Bacterial Branched-Chain Lipids

De novo fatty acid synthesis occurs in microbes utilizing one of two fatty acid synthetases (FAS-I and FAS-II). In eukaryotes, FAS-I is the enzyme complex used. This complex is characterized as having more limited product and primarily produces palmitic fatty acid. Prokaryotic *de novo* fatty acid synthesis utilizes FAS-II, which is more flexible in the primers recruited for fatty acid synthesis and therefore capable of producing a variety of fatty acids (Kaneda, 1967). Acetyl-CoA is the initiator for fatty acid synthesis and is converted to malonyl-CoA by acetyl-CoA carboxylase. The resulting malonyl-CoA is transferred to ACP by malonyl transacylase (FabD). β-ketoacyl-ACP synthase III (FabH) is primed by acyl-CoA and condenses with malonyl-ACP to produce β-ketoacyl-ACP. The following 4 reactions that occur elongate the β-ketoacyl-ACP. First, a condensing enzyme initiates each new round with malonyl-ACP, which is followed by the reduction to β-hydroxyacyl-ACP. This is followed by dehydration to *trans*-2-enoyl-ACP and the final reduction to complete one cycle of elongation. Repeated condensation increases the length by two carbon molecules per turn. The product of straight chain fatty acid synthase is a saturated straight chain fatty acid. The only difference among straight chain LCFA synthesis, OCFA synthesis, and BCFA synthesis is the primers used (Fulco, 1983; Kaneda, 1991). In the initiation phase of fatty acid synthesis, FabH in *Bacillus subtilis, Staphylococcus aureus,* and *Listeria monocytogenes* can introduce a isovaleryl-CoA, 2-methylbutyryl-CoA, or isobutyryl-CoA instead of acetyl-CoA; thus, the resulting BCFA have *iso* or *anteiso* branches at the terminal end (Annous et al., 1997; Choi et al., 2000;

Qiu et al., 2005). An example of pentadecanoic acid (C15:0) straight chain, *iso*, and *anteiso* is diagramed in Figure 2.6.

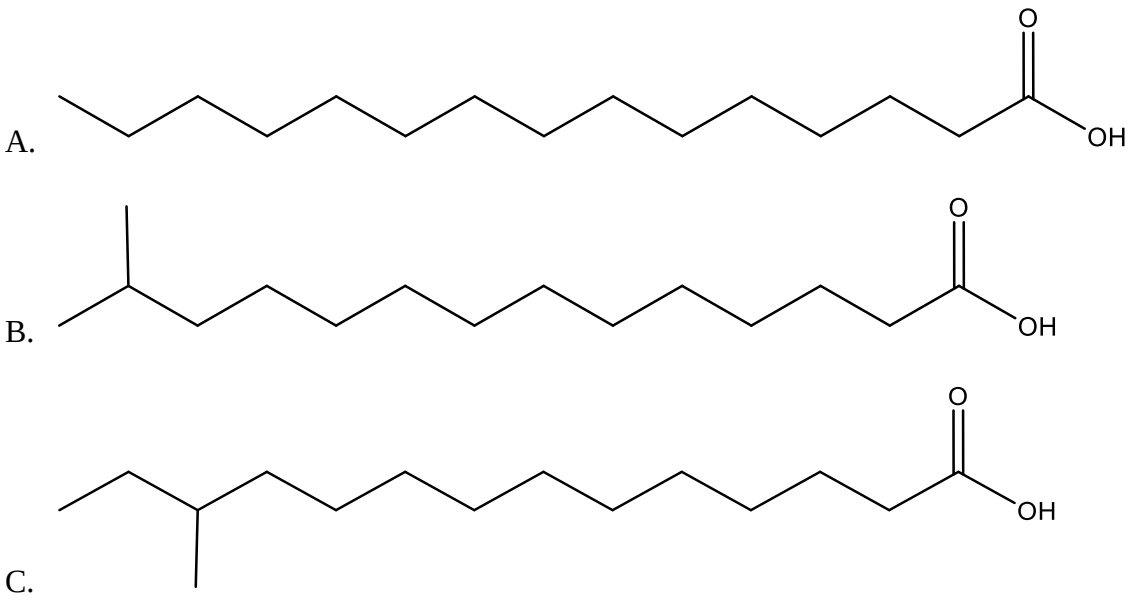

Figure 2.6 Pentadecanoic acid is shown as an example of a straight odd chain fatty acid (A), an iso fatty acid (B), and an anteiso fatty acid (C).

The change from straight chain fatty acid synthesis to BCFA is mediated by the substrate specificity of FabH in *Bacillus subtilis* and *Staphylococcus aureus* (Choi et al., 2000; Qiu et al., 2005; Zhang and Rock, 2008). This enzyme is shown to be temperature sensitive in *Listeria monocytogenes*, a Gram-positive bacterium known for its resistance to low temperatures. These bacteria incorporate a large proportion of *anteiso* fatty acids to

adapt to low temperatures (Annous et al., 1997). As temperature decreases, the FabH of *L. monocytogenes* increases its incorporation of 2-methylbutyrate (Singh et al., 2009). This change of substrate preference was not detected in the FabH of *E. coli*, but this bacterium adapts by desaturating saturated fatty acids and increasing incorporation of unsaturated fatty acids (Sinensky, 1971; 1974; Li et al., 2005). When FabH uses a 2-methylbutyryl-CoA primer, fatty acid synthesis yields *anteiso*-15:0 or *anteiso*-17:0; with isovaleryl-CoA, *iso*-15:0 or *iso* heptadecanoic acid (C17:0) are typically produced; and with isobutyryl-CoA as the primer, *iso* myristic acid (C14:0) and *iso* palmitic acid (C16:0) are synthesized (Allison et al., 1962b; Kaneda, 1977). The general pathway for synthesis of BCFA from BCVFA by rumen microbes is diagramed in Figure 2.7.

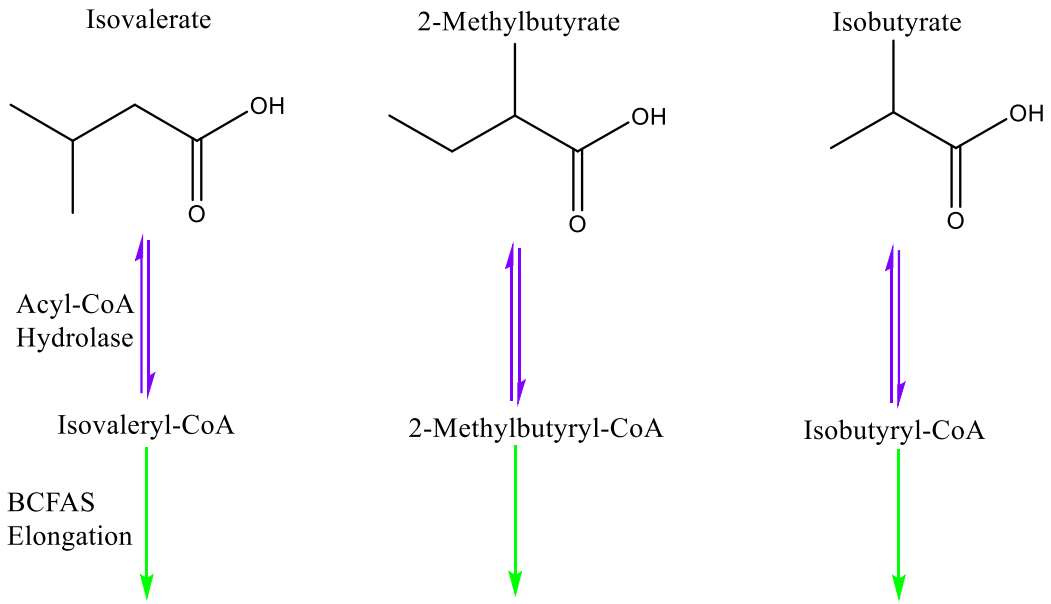

Figure 2.7 Synthesis of branched-chain fatty acids (BCFA) from branched-chain volatile fatty acids (BCVFA).
Where BCVFA are incorporated into BCFA by acyl-CoA hydrolase (purple) and branched-chained fatty acid synthase (BCFAS, green) and elongation by malonyl-CoA condensation in Bacillus spp. and Listeria monocytogenes (Kaneda, 1977; 1991; Annous et al., 1997).

Branched-chain volatile fatty acids primers are also recovered in plasmalogens. Allison et al. (1962b) documented that *Ruminococcus flavefaciens* incorporated ^{14}C from isovalerate (5 carbons) into C15 and C17 BCFA and a C15 aldehyde. In contrast, *R. albus* incorporated ^{14}C from isobutyrate (4 carbons) into C14 and C16 BCFA and aldehydes. The authors presumed that the aldehydes with the label were branched-chain aldehydes from plasmalogens. Plasmalogens, which are phospholipids with a vinyl ether at the sn-1 position, are formed by the reductive conversion of an ester bond in a phospholipid, which is different from the more well-known oxidative pathway in aerobic plasmalogen synthesis (Jackson et al., 2021). With acid hydrolysis the plasmalogen components are released and

the vinyl ether forms an aldehyde and during the methylation procedure it is converted to a DMA (Allison et al., 1962b). Whereas the released FA are methylated to form FAME. With GC analysis, individual DMA and FAME can coelute, which would underrepresent quantification of aldehyde because they are a much smaller proportion of bacterial lipids and have few commercial standards. These DMA can be relatively important products in mixed ruminal bacteria (Alves et al., 2013) and sink for BCVFA precursors (Allison et al., 1962b), but currently their role is not defined and needs further exploration (Goldfine, 2017).

Membrane fluidity and integrity are essential for normal and efficient function of rumen microbes. Desaturation of fatty acids is an aerobic process that is limited in the highly reduced rumen environment. Additionally, unsaturated fatty acids are typically biohydrogenated by the microbial population to prevent toxic effects (Hackmann and Firkins, 2015a). Thus, anaerobes can use either OCFA or BCFA to increase membrane fluidity, but it must be changed by incorporation of newly synthesized fatty acids in membranes rather than saturation/desaturation of existing fatty acids. Lower temperatures and higher pH would require greater membrane fluidity. Additionally, dietary conditions are likely to influence microbial membrane fatty acid profile.

Odd Chain Fatty Acids and Branched-chain Long Chain Fatty Acids Incorporation in Milk Fatty Acids

Odd-chain or BCFA are a distinct component of ruminant milk or meat products due to the microbial population in their rumens. There are few dietary BCFA available, and

even if all dietary BCFA were incorporated into milk, this portion would account for less than 10% of BCFA in milk fat. (Dewhurst et al., 2007; Vlaeminck et al., 2015). The interest in BCFA content of milk for consumers is due to their cytotoxic activity, which was comparable to CLA that have received considerable attention for their anti-cancer activity (Lock and Bauman, 2004; Vlaeminck et al., 2006). Current research with *iso*-15:0 has shown anti-cancer activity with cell cultures and mice (Yang et al., 2000; Wongtangtintharn et al., 2004; Cai et al., 2013). Retail milk has BCFA as a substantial fraction of milk fat profile; however, the estimated 400 mg/d of BCFA consumption from milk for consumers is much lower than that of the *iso*-15:0 treatments in the anti-cancer activity studies (Ran-Ressler et al., 2011).

The low supply of BCVFA that leaves the rumen and reaches the mammary gland is not incorporated into BCFA according to past studies. In a previous study with ^{14}C labeled isovaleryl-CoA, there was no selective incorporation of BCVFA to milk fat (Verbeke et al., 1959). Results from other studies that infused BCVFA or compared duodenal flow to milk fat BCFA also support the conclusion that BCFA are from microbial membranes and not *de novo* synthesis by the mammary gland (Croom et al., 1981; French et al., 2012; Vlaeminck et al., 2015; Prado et al., 2019). Though microbes are not the sole source of OCFA, they are considered the major supply for adipose or milk fat (Fulco, 1983). At the mammary gland straight chain FAS can use propionyl-CoA as the initial substrate, which yields odd chain lengths because the reactions initiate with a three-carbon substrate (Fulco, 1983; Kaneda, 1991). In theory, there is some *de novo* BCFA synthesis from methylmalonyl-CoA, the carboxylation product of propionate, which potentially accounts

for some higher milk secretion of BCFA than the reported duodenal flow (Horning et al., 1961; Smith, 1994; Dewhurst et al., 2007; Vlaeminck et al., 2015). However, the proportion of BCFA from *de novo* synthesis by the mammary gland from methylmalonyl-CoA was considered negligible by Croom et al. (1981). Another source for BCFA besides microbial membranes is mobilization from adipose tissue storage, which is more likely a source for higher levels of BCFA in milk compared to the flow in the duodenum. The BCFA mobilized still originates from microbial membranes. The BCFA can be altered post-ruminally by elongation (Vlaeminck et al., 2015; Prado et al., 2019). There is evidence that *anteiso*-13:0 is elongated to *anteiso*-15:0, *anteiso*-15:0 is elongated to *anteiso*-17:0, and *iso*-13:0 can be elongated to *iso*-15:0 before incorporation into milk fat (Prado et al., 2019). Therefore, increasing BCFA supply from microbial membranes can increase the concentration of bioactive fat in ruminant products (Bainbridge et al., 2018; Prado et al., 2019).

Branched-chain Amino Acids In Vitro and In Vivo Experiments

Studies investigating the effects of BCAA indicate that these amino acids influence microbial N and milk protein yield. When BCAA were removed from incubation media for mixed goat rumen microbes, microbial N from protozoa and bacteria decreased by 44.5% compared to the total essential amino acid treatment (Wang Meng-zhi, 2008). When buffered rumen fluid with wheat straw was supplemented with 0, 2, 4, 7, or 10 mmol/L of either Val, Leu, or Ile, total VFA production and NDF degradability increased (Zhang et

al., 2013). The response was greatest at 2 mmol/L of BCAA. Studies with BCAA abomasal or jugular infusions do not show consistent improvement in milk production, even when increases in blood BCAA levels were measured (Mackle et al., 1999; Korhonen et al., 2002; Appuhamy et al., 2011). However, Yoder et al. (2020) reported that jugular infused Leu and Ile did increase milk, energy-corrected milk, and protein yield. More consistently, when BCAA were removed from infusions of essential amino acids, milk protein yield decreased (Rulquin and Pisulewski, 2006; Haque et al., 2013; Doelman et al., 2015). In a study with late lactation Holstein multiparous cows, Hultquist and Casper (2016) fed Val (either 40 or 80 g/d), which increased milk and protein yield. When 40 g/d was supplemented, DMI increased, and when 80 g/d was supplemented, feed efficiency increased.

Increased milk protein from increased metabolizable BCAA supply could be mediated by amino acid stimulation of protein synthesis. Increasing levels of Leu and Ile increase rapamycin (mTOR) phosphorylation in bovine mammary cells, which partially mediates milk protein synthesis (Kim, 2009; Appuhamy et al., 2012). When all BCAA were removed from the infusion treatment by Doelman et al. (2015), mTOR complex 1 (mTORC1) was deactivated, and protein yield decreased compared to the total essential amino acid infusion. However, when Leu alone was removed from the infusion, protein yield was still low despite no indications of mTORC1 deactivation, suggesting that there is another mechanism mediating this response. Because infusions bypass the rumen, they do not see benefits in rumen function, which were reported with *in vitro* studies. However,

there are limited BCAA feeding studies, without rumen protected BCAA, to determine the effects of BCAA supplementation in dairy cattle.

Branched-chain Volatile Fatty Acids In Vitro and In Vivo Experiments

With isolated bacteria, some strains cannot catabolize BCAA or transport the provided amino acids, limiting their growth (Allison et al., 1962a). Thus, other researchers have focused on supplementation of BCVFA instead of BCAA to supply the growth factors more directly to the populations that require them. In an early *in vivo* study, valerate and isovalerate were supplemented to steers being fed straw (Hungate and Dyer, 1956). There were no changes in body weight or fermentation rate with BCVFA, but they hypothesized it was partially due to the lack of fermentable carbohydrates in the diet. Lassiter et al. (1958) recorded greater nitrogen retention, though no changes in production measurements, when isovalerate and valerate were supplemented *in vivo* with an alfalfa hay and corn silage diet. In mixed rumen bacteria incubated with timothy hay, addition of all BCVFA, isovalerate, or 2-methylbutyrate increased microbial protein synthesis, but isobutyrate or valerate alone did not (Russell and Sniffen, 1984). With supplemented ammonia salts of BCVFA and valerate microbial N increased *in vitro* with alfalfa and corn gluten meal plus urea as the crude protein (CP) source (Cummins and Papas, 1985). When ruminal contents were incubated with wheat straw, the addition of isovalerate, isobutyrate, or 2-methylbutyrate decreased ammonia concentration and increased plant cell wall degradation (Gorosito et al., 1985). The BCVFA did not act synergistically, and valerate

in this experiment did not change cell wall degradation or ammonia concentration. Valerate with BCVFA increased acetate and total VFA production in continuous cultures fed a diet consisting mainly of timothy hay and urea (Kone et al., 1989). Isobutyrate and isovalerate increased NDF degradation *in vitro* at 24 h with substrates consisting of alfalfa hay, pangolagrass, and napiergrass silage (Yang, 2002). With the addition of ammonium sulfate and supplemental isobutyrate or isovalerate, NDF degradation increased in the bermudagrass hay, alfalfa hay, pangolagrass, and napiergrass silage diets. Positive results both *in vitro* and *in vivo* indicate that the BCVFA with certain diets stimulated microbial growth and fiber degradation.

Dietary supplementation of BCVFA was a much more common study design than feeding studies with BCAA; therefore, there are more data from which to draw conclusions. In the 1980s, BCVFA research in dairy cattle was a notable area of interest. Felix et al. (1980a) supplemented isobutyrate, 2-methylbutyrate, isovalerate, and valerate, a combination referred to 'isoacids'. Different proportions of the BCVFA in the isoacid treatments were also explored. The isoacid mixture 1 was more balanced (80g/d, 28% isobutyrate, 24% 2-methylbutyrate, 24% isovalerate, and 24% valerate molar basis) and mixture 2 was not balanced (80 g/d, 36% isobutyrate, 17% 2-methylbutyrate, 17% isovalerate, and 30% valerate molar basis). Both mixtures increased milk production, milk persistency, and acetate molar proportion and decreased plasma urea nitrogen and rumen ammonia nitrogen. The same mixture treatments were used by Felix et al. (1980b) and improved nitrogen balance, and again the responses using the two mixtures did not differ from each other. Though Felix et al. (1980a) and Felix et al. (1980b) did not see any

differences between the 2 mixtures, this began an interest in determining an ideal isoacid blend for dairy cattle.

In following research studies Papas et al. (1984) utilized a study design that included 6 different isoacid blends, which were based on Felix et al. (1980a) and Felix et al. (1980b). The isoacid treatments consisted of 2 components: one was an even mix by weight of the 5 carbon acids (isovalerate, 2-methylbutyrate, and valerate) as ammonium salts and the second component had ammonium isobutyrate separate. Results from this study indicated that the blend (AS-VFA) with 31% isobutyrate, 25.2% 2-methylbutyrate, 19.4% isovalerate, and 24.4% valerate (anhydrous acid basis) had the best results for milk, protein, and total solids yields. The DMI was similar between treatment and the control; thus, the treatment improved feed efficiency. Peirce-Sandner et al. (1985) used the same AS-VFA blend (23.6 g/d isobutyrate, 19.2 g/d 2-methylbutyrate, 14.8 g/d isovalerate, and 18.6 g/d valerate anhydrous basis) and reported increased feed efficiency. However, when Klusmeyer et al. (1987) fed, ruminally infused, or abomasally infused an isoacid mix (25.7 g/d isobutyrate, 23.3 g/d 2-methylbutyrate, 17.8 g/d isovalerate, and 22.2 g/d) to post peak lactation cows, no changes in production, digestibility, or efficiency were noted.

Additionally, when a calcium salts isoacid mix was supplemented (IsoPlus^{TM} ; Eastman Chemicals, Kingsport, TN, USA), there were no changes in milk production (De Visser and Tamminga, 1987), pH (Robinson et al., 1987a), VFA concentration, and whole tract digestibility of nutrients (Robinson et al., 1987b). Calcium salts of BCVFA are solid instead of liquid like ammonium salts and benefit from reduced odor. In a study that included 43 commercial farms in 4 states, Rogers et al. (1989) supplemented either AS-

VFA (23.6 g/d isobutyrate, 19.2 g/d 2-methylbutyrate, 14.8 g/d isovalerate, and 18.6 g/d valerate) or IsoPlus™ (17.4 g/d isobutyrate, 15.1 g/d 2-methylbutyrate, 11.6 g/d isovalerate, and 13.9 g/d valerate). Milk fat, milk protein, and milk yields increased with supplementation, and the increase in milk yield was greatest with cows that were supplemented in early lactation than those introduced to the product later in the lactation. The greatest increase in milk yield was during the first 1 to 50 d of lactation. Differences between the ammonium salts and calcium salts isoacids were not reported. In another multi-site study at 4 university herds, Otterby et al. (1990) investigated a dosage response to BCVFA with the AS-VFA blend at 0, 0.4, 0.8, 1.2, or 1.6% inclusion in the concentrate portion of the diet throughout lactation. There was no difference in feed efficiency, but the 0.8% inclusion during mid (15.3 g/d isobutyrate, 12.4 g/d 2-methylbutyrate, 9.6 g/d isovalerate, 12 g/d valerate) and late (10 g/d isobutyrate, 8.2 g/d 2-methylbutyrate, 6.3 g/d isovalerate, 7.9 g/d valerate) lactation increased milk yield and protein yield. Another calcium salt isoacid product called ExtraLac™ (Continental Grain, Inc. Chicago, IL) was tested with a diet from which the CP source was feather meal and blood meal (Johnson et al., 1994). The cows were moved to the research diet at 28 to 35 DIM, but 90g/d of ExtraLac™ did not affect the acetate: propionate ratio, plasma urea nitrogen, DMI, or milk production.

From these studies, there were common factors that are hypothesized to influence the effectiveness of BCVFA supplementation. First, limitation of fermentable carbohydrates seems to limit any effects of BCVFA as demonstrated in steers fed straw by Hungate and Dyer (1956) or low starch diets such as the De Visser and Tamminga (1987)

study (1.2-5.4% DM) and in the study (8.3-8.4% DM) that was analyzed and reported by Robinson et al. (1987a) and Robinson et al. (1987b). Secondly, the stage of lactation affects the results of BCVFA supplementation as mentioned by Rogers et al. (1989) and Otterby et al. (1990). Though the 2 studies differed on what stage showed the greatest response, they also differed on when cows began supplementation. Rogers et al. (1989) did not begin supplementation of BCVFA at the same stage for all the cows in their 43-site study, but Otterby et al. (1990) began treatment 4 wk prior to parturition for all the cows in the study. The results from Otterby et al. (1990) do agree with those from Felix et al. (1980a) and Felix et al. (1980b) whereby cows that began BCVFA supplementation at < 40 DIM (1980a) or in early lactation (1980b) benefited from persistency of lactation. In comparison, Klusmeyer et al. (1991) did not begin their Latin square design study until the cows averaged 77 DIM. Therefore, supplementation prior to peak lactation or beginning in the prepartum period may be necessary for utilizing the full potential of additional BCVFA because that is when protein supplementation is most sensitive.

The nitrogen supply of the diets and amino acid profile of the protein sources is important to how the microbial population uses BCVFA. Johnson et al. (1994) reported no effects when the isoacid treatment was supplemented with blood meal (1.7% DM) and feather meal (5.0% DM) as the CP source. The diets were either 8% or 9.5% rumen-degraded protein (RDP), which was accomplished by adding urea in the higher RDP diet. The mixture of feather meal and blood meal has a different amino acid profile than plant-based protein sources and is notably lower in Ile (Cunningham et al., 1994). This may have caused an imbalance in the BCAA supplied to the rumen. The molar proportion reported

by Johnson et al. (1994) was 0.5 mol/100 mol for 2-methylbutyrate and isovalerate and isobutyrate was 0.7 mol/100 mol without BCVFA supplementation in the rumen. When comparing these molar proportions to other studies isovalerate and 2-methylbutyrate ranged from 1.06 to 1.61 mol/100 mol and isobutyrate ranged from 0.75 to 1.35 mol/100 mol (Liu et al., 2009a; Liu et al., 2009b; Wang et al., 2012; Liu et al., 2018a; Wang et al., 2018; Wang et al., 2019). The molar proportion of 2-methylbutyrate and isovalerate reported by Johnson et al. (1994) was considerably lower compared to other studies, but isobutyrate was closer to other reported values. This study, like numerous others, did not separate isovalerate or 2-methybutyrate and they were combined in the analysis of the rumen VFA profile. Both feather meal and blood meal resist degradation in the rumen which could have limited nitrogen supply to the microbial population. The requirement of adequate nitrogen supply of BCVFA usage was demonstrated by Yang (2002) with the bermudagrass diet. This was the lowest CP diet (4.7% CP on DM basis) in the experiment, so when isovalerate or isobutyrate was supplemented, NDF degradation did not change. However, when ammonium sulfate was added to the buffer, supplemented isovalerate or isobutyrate increased NDF degradability from 16.2% to 21.0% and 19.3%, respectively.

After these studies, research on BCVFA supplementation decreased, likely due to potential palatability and handling issues because of the smell of the isoacid mixtures (Rosener and Uhlenhopp, 1987). Research on BCVFA returned during the 2000s. These studies differed because researchers more commonly supplemented a single BCVFA to investigate the responses to the individual supplement rather than the isoacid combination. Liu et al. (2009b) supplemented Chinese Jinnan Yellow × Holstein cows with isobutyrate

at 4 levels (0, 20, 40, 60 g/d) to measure the effects with a corn silage (26%), corn gain (23%), corn stover (21%) diet with 16.3% CP. Isobutyrate increased milk yield, and the greatest yield was at 40 g/d without affecting DMI. Isobutyrate linearly decreased rumen pH, ammonia nitrogen, and plasma non-esterified fatty acids (NEFA), but rumen VFA concentration, acetate:propionate, and plasma growth hormone (GH) concentration increased linearly. However, in this same study, milk fat and protein percentage and yield were quadratically related to isobutyrate dosage and were the lowest at 20 g/d. Milk fat content also tended to decrease linearly. In another study with only supplemental isobutyrate, Simmental steers were fed a diet that was 60% corn stover, 21% ground corn grain, and 10% CP diet were given either no isobutyrate, 8.4g/d, 16.8 g/d, or 25.2 g/d (Wang et al., 2015). Quantification using qPCR showed linear and quadratic increases in the abundance of bacteria, fungi, and protozoal. The greatest values were reported with 16.8 g/d of supplementation. Methanogens and methane production decreased linearly. Also, the specific targets *Ruminococcus albus, R. flavefaciens, Butyrivibrio fibrisolvens,* and *Fibrobacter succinogenes* increased linearly with isobutyrate supplementation. The enzyme activities of carboxymethylcellulase, α-amylase, xylanase, β-glucosidase, and α-glucosidase, also increased linearly. Only urease and protease activity decrease linearly.

These studies were not designed to measure the same responses and used very different dosages and animal models. However, both have responses that indicate isobutyrate stimulated microbial function and growth in a dose-responsive manner. Liu et al. (2009b) recorded post ruminal effects of isobutyrate such as decreased component yield and plasma concentrations of NEFA, whereas glucose, and GH plasma concentrations

increased linearly (Liu et al., 2009b). Higher concentrations of GH can increase milk fat, protein, and lactose, but Liu et al. (2009b) did not report those results (McDowell et al., 1987). As mentioned before, the earlier lactation supplementation began, the greater the effects of BCVFA were, so Liu et al. (2009b) may have supplemented cows too late to see the full benefits of isobutyrate in milk production (Rogers et al., 1989; Otterby et al., 1990).

When Simmental steers fed a corn stover diet were supplemented with 0, 8.4, 16.8, or 25.2 g/d of 2-methylbutyrate, rumen pH and ammonia nitrogen concentration decreased linearly (Wang et al., 2012). Ammonia nitrogen also showed a quadratic relationship to 2-methylbutyrate dose because the concentration decreased more rapidly with higher levels of supplementation. Rumen VFA concentration; acetate:propionate; activities of xylanase, carboxymethylcellulase, and protease; and total purine derivatives all increased linearly and quadratically because the differences were less at higher dosages than at the lower doses. Zhang et al. (2015) used the same treatments and diet in Simmental steers in a follow-up study and measured a linear increase in anaerobic fungi, protozoa, bacteria, *R. albus, R. flavefaciens*, and *Fibrobacter succinogenes* 16S rRNA gene copies as quantified by specific qPCR. However, methanogens decreased linearly. The activity of carboxymethylcellulase, α-amylase, xylanase, β-glucosidase, and α-glucosidase also increased linearly. Urease and protease activity decreased linearly. Methane production decreased linearly with increasing 2-methylbutyrate supplementation. In another 2-methylbutyrate study, Holstein bull calves starting at 15 d of age were fed either 0, 3, 6, or 9 g/d of 2-methylbutyrate (Liu et al., 2016). After 31 d of age ADG increased linearly and feed conversion ratio (kg DMI/ADG) decreased linearly (i.e., improved). At 90 d of age,

2-methylbutryate affected rumen development and increased total stomach, rumen weight, papillae length, and papillae width linearly. At 90 d of age, 2-methylbutyrate linearly increased plasma concentrations of β-hydroxybutyrate (BHB), GH, and insulin-like growth factor 1 (IGF-1). In another study with Simmental steers, 0 or 15 g/d of 2-methylbutyrate was supplemented with either a moderate (40%) or a high (60%) concentrate diet (Wang et al., 2018). In the moderate diet, 2-methylbutyrate decreased rumen ammonia nitrogen and pH, but total VFA and acetate:propionate increased. In the high concentrate diet, the acetate:propionate and pH decreased (6.69 versus 6.35), but 2-methylbutyrate supplementation did not affect these measurements with this diet. The relative sequence abundances of *R. albus, R. flavefaciens, F. succinogenes, B. fibrisolvens, Prevotella ruminicola*, and *Ruminobacter amylophilus* increased with 2-methylbutyrate in the moderate concentrate diet. The high concentrate diet decreased the relative sequence abundance of *R. albus, R. flavefaciens*, *F. succinogenes*, and *B. fibrisolvens,* whereas *P. ruminicola* and *R. amylophilus* increased. The high concentrate diet also decreased the activities of carboxymethylcellulase, cellobiase, xylanase, pectinase, and protease. When 2-methylbutyrate was supplemented with a moderate concentrate diet, these enzyme activities increased. Urinary excretion of total purine derivatives and allantoin increased with the high concentrate diet and when 2-methylbutyrate was supplemented in the moderate concentrate diet, suggesting increased ruminal production of microbial protein.

General conclusions from these studies are that 2-methylbutyrate consistently increased microbial enzyme activity and that cattle benefited from increased VFA concentration and microbial N (Wang et al., 2012; Zhang et al., 2015; Liu et al., 2016;

Wang et al., 2018). Fiber-degrading bacteria especially benefited as indicated by the acetate:propionate ratio and abundance. There were also benefits for rumen development in calves. Because of greater VFA production, butyrate increased, which stimulates rumen development and increased BHB (Liu et al., 2016). As a result, GH and IGF-1 concentrations increased, which should have increased protein synthesis and reduced protein oxidation in body tissues and explaining their greater ADG (Brameld et al., 1996; Breier, 1999). As noted by Wang et al. (2018), 2-methylbutyrate benefits were greater in the moderate concentrate diet compared to the high concentrate diet. The high concentrate diet had different substrate availability and lower pH, which altered the rumen microbiome and environment, which decreased the demand and benefit of supplemental 2-methylbutyrate. It is not clear if the difference in response to 2-methylbutyrate with the different diets is due to pH, because a lower pH environment requires less BCFA for membrane fluidity (especially 2-methylbutyrate), or the difference in the microbial populations because the populations of bacteria that require BCVFA decreased.

In cannulated Simmental steers fed the corn stover diet, isovalerate was dosed at either 0, 100, 200, or 300 mg/kg DM (Liu et al., 2009a). Isovalerate linearly increased rumen total VFA concentration and acetate:proprionate. These measurements also responded quadratically to isovalerate and the highest values were recorded at 200 mg/kg DMI. Isovalerate linearly decreased pH and ammonia-N. Dose linearly increased and had a quadratic effect on excretion of allantoin and total purine derivatives, and total tract digestibilities of NDF and acid detergent fiber (ADF). The highest measurements were also recorded at 200 mg/kg DMI. Liu et al. (2014) continued investigating isovalerate with

cannulated Simmental steers that were dosed with either 0, 8.4, 16.8, or 25.2 g/d of isovalerate with the corn stover diet. The 16S rRNA gene copies/mL of *Ruminococcus albus*, *R. flavefaciens*, *Butyrivibrio fibrisolvens*, and *Fibrobacter succinogenes* increased linearly with increasing dosage of isovalerate as did the activity of carboxymethylcellulase, α-amylase, xylanase, β-glucosidase, and α-glucosidase. Whereas urease and protease activity decreased linearly. Methane production and total methanogen decreased. Isovalerate was also investigated in dairy bull calves (Liu et al., 2018c). Isovalerate supplemented at either 0, 3, 6, or 9 g/d linearly increased rumen weight, body weight, papillae length, papillae width, GH receptor mRNA in rumen mucosa, IGF-1 receptor mRNA in rumen mucosa, plasma BHB, plasma GH, and plasma IGF-1 at 90 d of age.

Like the other individual BCVFA investigated, isovalerate demonstrated multiple benefits such as stimulation of some microbial populations so that animals benefited from increased microbial N and microbial enzyme activity (Liu et al., 2009a; Liu et al., 2014; Liu et al., 2018c). Again, these responses seemed most related to fiber-degrading bacteria, as demonstrated by the acetate:propionate ratio and NDF and ADF digestibilities. Isovalerate also stimulated rumen development; with the additional energy provided to the calves blood concentrations of GH and IGF-1 increased as did GH receptor mRNA, and IGF-1 receptor mRNA in the rumen mucosa (Liu et al., 2018c). These results explain the increased body weight in supplemented calves.

All the studies with individual BCVFA treatments had numerous benefits such as increased microbial N and bacterial abundance measured by qPCR quantification of rumen bacterial targets, urinary excretion of purine derivatives and/or allantoin, and decreased

rumen ammonia nitrogen concentration (Liu et al., 2009a; Wang et al., 2018). Supplemental BCVFA increased acetate:propionate and total VFA concentration, which decreased rumen pH (Liu et al., 2009a; Liu et al., 2009b; Wang et al., 2012; Wang et al., 2018). Methanogens and methane production were decreased with isobutyrate (Wang et al., 2015), 2-methybutyrate (Zhang et al., 2015) and isovalerate (Liu et al., 2014) supplementation. Because at least some methanogens also require BCVFA (Hobson and Stewart, 1988), such results suggest a shift away from H_2-producing bacteria or simply a result of lower ruminal pH described previously. Also, decreased methanogenesis could be a result of providing BCVFA instead of BCAA; BCAA and BCFA synthesis from BCVFA can serve as an alternate hydrogen sink and therefore decrease the H_2 available for methanogenesis. The results of the feeding studies were very consistent with each BCVFA, most likely because the diet in many of the studies was corn stover, which was low in CP and therefore making BCVFA more beneficial. Liu et al. (2009b) was the only study with lactating cows, and although isobutyrate increased milk yield, milk fat and protein concentration and yield decreased, suggesting isobutyrate alone might not be optimal for late lactation cows. This demonstrates that there are potential post-rumen effects of BCVFA that require further investigation. Isovalerate (Liu et al., 2018c) and 2-methylbutyrate (Liu et al., 2016) also saw post rumen effects, though this was demonstrated in dairy bull calves during rumen development.

During the late 2010s, studies with supplementation of all BCVFA were more common. Liu et al. (2018a) supplemented Holstein cows at 65 DIM with 0, 30, 60, or 90 g/d of BCVFA (1: 1: 1 of isobutyrate: 2-methylbutyrate: isovalerate). The diet was corn

silage (27%), corn grain (23%), corn stover (20%), and the remainder as concentrate. The diet had 16% CP. Like the individual BCVFA studies, total VFA concentration and acetate: propionate increased linearly and quadratically with increasing BCVFA supplementation, whereas pH (linear and quadratically) and rumen ammonia nitrogen (linear) decreased. Total tract apparent digestibilities of organic matter (OM), CP, ether extract (EE), NDF, and ADF increased linearly. Milk production increased linearly with increasing BCVFA. Milk fat and protein concentration and yield also increased linearly. The FCR (kg DMI/kg milk yield) decreased linearly. The milk fat decreased linearly in C16-22 fatty acids with increasing BCVFA dose, potentially resulting from the increased *de novo* fatty acid synthesis at the mammary gland. This study was unique in that it measured mRNA expression of fatty acid synthesis genes in the mammary gland. Gene expression of peroxisome-proliferator-activated receptor γ (PPARγ), sterol regulatory element-binding factor 1 (SREBF1), and fatty acid-binding protein 3 (FABP3) increase linearly and quadratically such that cows fed the 60 g/d treatment had the highest expression. Acetyl-coenzyme A carboxylase α (ACC), FAS, and stearoyl-CoA desaturase (SCD) increased linearly, but lipoprotein lipase (LPL) expression was not affected. In dairy bull calves, 0, 60, 120, 180 mg/d of BCVFA (1: 1: 1) supplementation started at 10 mo of age. BCVFA increased DMI, final weight, and ADG linearly, but the FCR (kg DMI/ADG) decreased linearly (Liu et al., 2018b). Again, ruminal pH decreased linearly, but total VFA concentration and acetate:propionate ratio increased linearly with increasing BCVFA supplementation. Total tract apparent digestibilities of DM, OM, CP, EE, NDF, and ADF increased linearly. Dose tended to linearly increase plasma concentrations of glucose, NEFA, and albumin increased, but BHB decreased. Triglyceride concentration of the liver

tended to decrease linearly, whereas total lipid content did not change. For liver mRNA expression, peroxisome proliferator-activated receptor α (PPARα) and carnitine palmitoyl transferase-1 (CPT1) increased linearly, but SREBF1, ACC, and FAS decreased linearly with increasing BCVFA dose. Finally, in a study with Holstein cows at 65 ± 2.6 DIM were used in a 2 × 2 factorial study (Wang et al., 2019). The factors were 0 or 80 g/d of BCVFA (1: 1: 1) and 0 or 128 mg/d of folic acid. The cows in this study were fed a diet that consisted of ensiled corn fodder (26%), ground corn grain (24%), alfalfa hay (14%), soybean meal (14%), and oat hay (10%), and it had 16.6% CP. Additional BCVFA increased DMI, milk production, rumen total VFA concentration, acetate:propionate, and decreased FCR (kg DMI/kg milk yield), pH, and rumen ammonia nitrogen. Total tract apparent digestibilities of OM, CP, non-fiber carbohydrate (NFC), NDF, and ADF increased with BCVFA. The activity of carboxymethylcellulase, cellobiase, xylanase, pectinase, α-amylase, and protease increased with BCVFA. The relative 16S rRNA gene sequence abundance of *Ruminococcus albus*, *R. flavefaciens*, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, and *Prevotella ruminicola* increased with BCVFA supplementation. In this study, plasma glucose and NEFA did not change with supplemental BCVFA, but total protein, ACC, BHB, growth hormone-releasing hormone (GHRH), GH, and IGH-1 plasma concentrations increased.

The increase in VFA concentration, decreased in rumen pH, and decrease in rumen ammonia nitrogen that was consistent with individual BCVFA supplementation also occurred when all 3 were dosed in lactating dairy cattle (Liu et al., 2018a; Wang et al., 2019). They reported increased apparent digestibility of nutrients and improved feed

efficiency. Milk fatty acid profile with BCVFA supplementation has not been measured in previous studies, but Liu et al. (2018a) saw a linear decrease in C16-22, which could indicate increased *de novo* fatty acid synthesis at the mammary gland. This is supported by the increased PPARγ and SREBF1 mRNA in bovine mammary gland. These genes regulate ACC, FAS, SCD, FABP3 and LPL, which all increased with BCVFA supplementation except for LPL (Bernard et al., 2008). Increases in ACC and FAS would increase *de novo* fatty acid synthesis, which would increase the composition of <16C fatty acids. FABP3 and LPL transport LCFA and regulate uptake at the mammary gland (Lehner and Kuksis, 1996). Liu et al. (2018a) also only separated saturated (C4-C15), 14:1, 15:1, and 16-22 for the milk fatty acid profile and did not separate BCFA or biohydrogenation intermediates. The dairy bull calf study did investigate changes in fat metabolism in the liver, which was not in the previous research with BCVFA (Liu et al., 2018b). The effect of BCVFA on hepatic mRNA expression matched the response of acetate-treated bovine hepatocytes *in vitro*; acetate regulated gene expression of lipid metabolism genes via activation of the adenosine monophosphate activated protein kinase signaling pathway (Li et al., 2013). The responses of the acetate treated hepatocytes matched BCVFA supplemented steers because BCVFA increased acetate due to increased ruminal VFA concentration and molar proportion of acetate (Liu et al., 2018b). As a result, liver triglyceride content decreased even with higher NEFA plasma concentrations. The lower triglyceride levels could be a reason why BCVFA supplementation benefits cows in early lactation more than middle or late lactation because during the transition period cows are most at risk for fatty liver (Grummer, 1993). The lactating cows that were studied by Liu et al. (2018a) and Wang et

al. (2019) were not in early lactation (65 DIM), but they noted improved feed efficiency and milk production from BCVFA supplementation.

Chapter 3. Assessing milk response to different combinations of valerate and branched chain volatile fatty acids in primiparous and multiparous Jersey cows

Introduction

The diverse rumen microbiota degrades nutrients in a consortium that relies on cross-feeding (Firkins, 2021). Branched-chain VFA (BCVFA), which are formed by the degradation of branched-chain AA (BCAA), are required growth promoting factors for some fiber-degrading bacteria (Allison et al., 1962a; Allison et al., 1962b). Catabolism of Ile, Val, and Leu forms 2-methylbutyrate (MB), isobutyrate (IB), and isovalerate (IV), respectively. Some bacterial strains are incapable of degrading BCAA (Allison, 1978) and depend on cross-feeding to uptake BCVFA for direct BCAA synthesis or primers for branched-chain long chain fatty acid (BCFA) synthesis (Allison et al., 1962b; Robinson and Allison, 1969).

Interest in BCVFA (colloquially called ‘isoacids’) as growth-promotors of rumen microbes in dairy cattle lead to the development of IsoPlus^{TM} (Eastman Chemicals, Kingsport, TN, USA), which was a mix of the 3 BCVFA and valerate. Valerate has commonly been included in these supplement mixtures because it was a growth factor for some strains of bacteria. The most consistent benefits of isoacid supplementation in numerous multi-location trials were either increased milk production with similar DMI or

increased feed efficiency (Papas et al., 1984; Peirce-Sandner et al., 1985). Limiting factors included potential palatability and handling issues due to the unpleasant smell of the isoacid mixture (Rosener and Uhlenhopp, 1987). Palatability concerns could not be distinguished from a potential physiological limitation on voluntary DMI. Despite lower DMI, milk production was unchanged and resulted in improved milk efficiency.

Many of the earlier studies had different proportions of the 4 isoacids, whereas the later studies typically supplemented only a single BCVFA or all 3 without determining which combinations of BCVFA or valerate were necessary to elicit positive responses. Feeding IV (Liu et al., 2014), IB (Wang et al., 2015), or MB (Wang et al., 2018) demonstrated similar benefits even when supplemented individually and increased activity of enzymes for a range of substrates (including xylanase and carboxymethylcellulase activities) and abundance of fibrolytic bacteria. When evaluating various combinations of BCVFA, Roman-Garcia et al. (2021a) concluded that MB combined with either IB or IV was the most beneficial combination for NDF digestibility in batch cultures of mixed rumen microbes. The optimal BCVFA mixture may be diet-dependent, such that diets containing large amounts of rumen degradable Leu (i.e., corn-based diets) would be less likely to benefit from IV supplementation. Additionally, Roman-Garcia et al. (2021a) failed to detect responses to valerate supplementation in multiple studies but repeatedly emphasized MB followed by IB as the most beneficial BCVFA. Therefore, our objective was to determine the combination of isoacids, stepping down from all 3 BCVFA plus valerate (mimicking IsoPlus) to MB + IB to MB alone compared to a control to discern, that would be optimal for milk production and/or milk efficiency in Jersey cattle. We

expected that supplementation of isoacid or MB + IB will increase feed efficiency compared to the control, whereas the cows only supplemented with MB will increase feed efficiency but to a lesser extent.

Material and Methods

Experimental design and treatments

Sixty Jersey cows (28 primiparous and 32 multiparous; 106 ± 54 DIM) were blocked into 15 blocks of 4 cows each according to parity, days in lactation, and daily milk production. Cows were housed and managed according to Institutional Animal Care and Use Committee approved protocol at The Ohio State University's Waterman Dairy in Columbus. Within a block, cows were randomly assigned to 4 treatments: Control (**CON**) treatment without any isoacids, MB (12.3 mmol/kg DM of 2-methylbutyrate), MB + IB (7.7 and 12.6 mmol/kg DM, respectively, of MB and isobutyrate), or ISO (6.2, 7.3, 4.2, and 5.1 mmol/kg DM, respectively, of MB, IB, IV, and valerate). The ISO dosage was based on the original research in the development of IsoPlus^{TM} (Eastman Chemicals, Kingsport, TN, USA) containing valerate and the 3 BCVFA (Papas et al., 1984; Peirce-Sandner et al., 1985; Otterby et al., 1990). The ISO treatment was most similar to that of Otterby et al. (1990), who used a range of dosages for different stages of lactation and did not detect an increased response to isoacids above the 0.8% of concentrate treatment, which was approximately 5.9 to 9.9, 8.4 to 14.1, 4.5 to 7.6, and 5.7 to 9.6 mmol/kg DM of MB,

IB, IV, and valerate, respectively. Additionally, the ISO treatment was utilized by Copelin et al. (2021). The MB + IB treatment was calculated from the Nutritional Dynamic System, which uses the Cornell Net Carbohydrate and Protein System platform. The MB dose (7.7 mmol/kg DM) was comparable to that in the ISO treatment (6.2 mmol/kg DM), and the IB dose (12.6 mmol/kg DM) was increased to approximately the sum of IB and IV (11.5 mmol/kg DM) in the ISO treatment. The dose for the MB treatment (12.3 mmol/kg DM) was approximately the same as the dose of IB (12.6 mmol/kg DM) in the MB + IB treatment. Roman-Garcia et al. (2021a) documented the additive effects of individual BCVFA to improve NDF degradability in vitro and for IB to be able to substitute for IV. The reported doses herein are based on analyzed values, explaining slight deviations from the formulated doses and their justifications.

All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee. The cows were moved into the tie-stall barn at the Waterman Dairy Complex, which had individual stalls and mangers. All cows were fed the CON diet for the first 2 wk, with wk 1 allowing for adaptation to the tie stalls and wk 2 serving as a covariate period. Starting on wk 3 and continuing through the end of wk 10, cows were fed their respective treatment diets (Table 3.1). The diets were formulated to contain 16.0% CP for all treatments, including 0.29% urea being added to provide sufficient ruminal ammonia concentration (Spartan Ration Evaluator/Balancer for Dairy Cattle, v3, Michigan State University, East Lansing). The BCVFA were supplemented as Mg salts (Zinpro Corporation, Eden Prairie, MN) and replaced a proportion of the wheat middlings in the MB, MB+ISO, and ISO concentrate pellet mixes. The supplements and pellets were

combined, and composite samples were used to define average doses. Both samples of the BCVFA supplements (10 g/ 250 mL) and concentrate pellets (25 g/ 250 mL) were soaked in distilled water for 24 h before being processed and analyzed by GLC, as described by Roman-Garcia et al. (2021a). The only difference was that 0.2 mL or 0.1 mL of sample from concentrate or supplement samples, respectively, were diluted with 1 mL of distilled water instead of 0.4 mL of sample to account for the more concentrated BCVFA in feed concentrate samples.

Cows were milked twice daily at 0500h and 1700h. Fresh TMR was mixed and fed at approximately 110% of ad libitum intake once per day. After milking, the cows were returned to their respective stalls, and half of the fresh TMR was fed to the animals. The remaining feed was offered the following morning after milking. Feed was pushed up twice daily. All stalls had access to automatic waterers.

Sample Collection, Processing, and Analyses

Ingredients were sampled weekly, and a subsample was dried at 55 °C overnight for DM analysis and used to maintain the proportions of the ingredients in the diet. Samples of the TMR, prior to feeding, were also taken on a weekly basis, and individual ingredients and TMR samples were composited over the 2 wk of the covariate period and every 4 wk thereafter for determination of chemical composition by Cumberland Valley Analytical Services (Waynesboro, PA). Dry matter, ash-free NDF (using amylase and sulfite, Van Soest et al., 1991), starch (Hall, 2009), CP (combustion method), ADF, ash, Ca, P, Mg, K,

Na, Fe, Mn, and Zn were determined using wet chemistry analyses (AOAC, 1990; methods 990.03, 973.18, 942.05, 985.01, respectively).

Body condition was assessed by 2 trained individuals and averaged at the beginning of wk 1, 3, and 7 and at the end of wk 10. After milking and before returning to their stalls, the cows' BW was recorded once every week. The BW from wk 3 to 10 were regressed, and the linear slope was the ADG variable used for each cow.

Milk weights were recorded by integrated meters during each milking (Afimilk Agricultural Cooperative Ltd., Afikim, Israel). Each week, samples from 4 consecutive milkings were collected and sent to Dairy Herd Improvement Cooperative Inc. (DHI, Columbus, OH) for determination of true protein, milk fat, lactose (B2000 Infrared Analyzer; Bentley Instruments, Chaska, MN), and MUN (Skalar SAN Plus segmented flow analyzer; Skalar Inc., Norcross, GA) content. During wk 5 and 9, a sample from the morning and evening milking for 2 d was collected and pooled. The sample was centrifuged at 14,000 × *g* at 4°C for 30 min. The 2 daily milk fat cakes were pooled within week and methylated as described by Jenkins (2010), using the modifications to determine CLA isomers in milk fat. The internal standard used was 17:1 *cis*-10 (Sigma-Aldrich, Laramie, WY), which was used to prevent coelution with bacterial fatty acids (FA) recovered in the milk compared with alternate 19:0 and 17:0 internal standards used in other publications.

The fatty acid methyl esters (FAME) were separated by GC (HP 5890 series, Agilent Technologies, Santa Clara, CA) using a SP-2560 capillary column (100 m × 0.25 mm × 0.2-μm film thickness; Supelco, Bellefonte, PA). The carrier gas (He) flow was 20

cm/s, inlet temperature was 250°C, and the detector temperature was 250°C. The split was 60:1; a lower split compared with other publications was chosen to identify smaller peaks from BCFA in the milk fat samples. The initial oven temperature was 60°C and was held for 4 min, followed by a 2°C/min increase to 140°C, which was held for 30 min. The temperature was then increased by 1°C/min to 160°C and held for 60 min. After the 18:1 isomers were eluted, the temperature was increased at 1°C/min to 190°C and held for 60 min to prevent carryover. Individual FAME were identified using the standard mix GLC-68D (Nu-Check Prep Inc., Elysian, MN), GLC-110 (Matreya LLC, State College, PA), and the single FAME standards 18:1 *trans*-11 and 18:2 *trans*-10, *cis*-12 (Nu-Check Prep Inc., Elysian, MN). Then standard mixes from Bacterial Acid Methyl Esters CP Mixture (Matreya LLC, State College, PA) and 37 Component FAME Mix (Sigma-Aldrich, Laramie, WY) were used for peak identification and response factor calculations. Chromatographs from Molkentin and Precht (1995) were used for the order of elution of 18:1 isomers. Milk FA yields and profiles were calculated with the equations described by Glasser et al. (2007) and applied as described by Rico and Harvatine (2013).

Statistical Analysis

During the study, 1 multiparous cow and 1 primiparous cow, both on the MB + IB treatment, were removed from analysis due to high SCC. Responses were averaged by week except DMI and efficiency measurements, which were averaged biweekly (DMI, N intake, NE_L/DMI, Milk N production/N intake) to smooth out the repeated measures

responses. The ADG calculation was not averaged over time because it was derived as a slope response of BW over time in the 8-wk treatment period and did not have a covariate.

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

$$Y_{xijtn} = \mu + Cov_x + S_i + P_j + W_t + (S \times P)_{ij} + (S \times W)_{it} + (P \times W)_{jt} + (S \times P \times W)_{ijt} + b_n + c_{x:jn} + e_{xijtn},$$

where Y_{xijtn} is the dependent variable; μ is the overall mean, Cov_x is the covariate for cow x (x = 1 to 58), S_i is the fixed effect of treatment (i = 1 to 4; CON, MB, MB + IB, or ISO), P_j is the fixed effect of parity (j = 1 or 2, primiparous or multiparous), W_t is the fixed effect of time, where t = 3 to 10 weekly measurements or t = 3.5, 5.5, 7.5, and 9.5 biweekly measurements, $(S \times P)_{ij}$, $(S \times T)_{iw}$, $(P \times T)_{jw}$, $(S \times P \times T)_{ijw}$ are the respective interactions, b_n is the random effect of block (n = 1 to 15), $c_{x:jn}$ is the random effect of subject n within treatment i and parity j, and e_{xijtn} is the random error. Denominator degrees of freedom were derived using the Kenward-Roger adjustment. Data were analyzed using repeated measures (weekly or biweekly) with the first-order autoregressive covariance structure, which generally had the lowest Akaike Information Criterion among variables. Results are reported as the least squares means and pooled standard error of the mean for the main effect of treatment for all data. If there was an interaction with time, treatment means were compared using LSMESTIMATE. Differences and trends were declared at $P \leq 0.05$ and $P \leq 0.10$, respectively.

Table 3.1. Ingredient and nutrient composition (with standard deviations) of diets containing different combinations of branched-chain volatile fatty acids

Composition	CON[1]	MB	MB+IB	ISO
Ingredient, % DM basis				
Alfalfa haylage	14.8	14.8	14.8	14.8
Corn silage	37.8	37.8	37.8	37.8
Brewer's grains	8.21	8.21	8.21	8.21
Concentrate pellet				
DCAD Plus™ [2]	0.098	0.098	0.098	0.098
Dried molasses	1.00	1.00	1.00	1.00
Wheat middlings	5.16	4.62	4.37	4.37
All isoacids supplement [3]	—	—	—	0.798
IB supplement [4]	—	—	0.457	—
MB supplement [5]	—	0.545	0.341	—
Ground shelled corn	18.2	18.2	18.2	18.2
Megalac® [2]	1.43	1.43	1.43	1.43
AminoPlus® [6]	9.65	9.65	9.65	9.65
Urea	0.286	0.286	0.286	0.286
Sodium bicarbonate	0.655	0.655	0.655	0.655
Dicalcium phosphate	0.293	0.293	0.293	0.293
Limestone	0.905	0.905	0.905	0.905
Potassium chloride	0.629	0.629	0.629	0.629
Magox® [7]	0.131	0.131	0.131	0.131
Dynamate® [8]	0.169	0.169	0.169	0.169
White salt	0.238	0.238	0.238	0.238
Trace-mineral premix [9]	0.150	0.150	0.150	0.150
Biotin 100	0.036	0.036	0.036	0.036
Vitamin mix [10]	0.176	0.176	0.176	0.176
Nutrient, % DM basis				
DM, % as is basis	43.2 ± 2.8	43.2 ± 2.8	43.4 ± 2.7	43.3 ± 2.6
CP	15.8 ± 0.4	15.9 ± 0.5	16.0 ± 0.6	16.0 ± 0.7
ash-free NDF	32.5 ± 0.4	30.9 ± 1.1	30.7 ± 1.0	31.7 ± 1.3
ADF	18.0 ± 0.7	17.5 ± 0.7	17.0 ± 0.7	18.6 ± 1.9
Starch	26.3 ± 0.5	26.4 ± 0.6	26.5 ± 0.4	26.0 ± 0.7
Ca	0.99 ± 0.03	1.10 ± 0.07	1.13 ± 0.05	1.12 ± 0.05
P	0.47 ± 0.02	0.45 ± 0.03	0.45 ± 0.03	0.45 ± 0.03
Mg	0.33 ± 0.01	0.39 ± 0.03	0.39 ± 0.02	0.42 ± 0.03
K	1.45 ± 0.02	1.46 ± 0.04	1.45 ± 0.05	1.45 ± 0.05
Na	0.33 ± 0.02	0.33 ± 0.02	0.33 ± 0.02	0.34 ± 0.01
Fe, mg/kg	188 ± 9	202 ± 16	203 ± 19	193 ± 27
Mn, mg/kg	98 ± 7	95 ± 16	90 ± 9	96 ± 10
Zn, mg/kg	115 ± 11	130 ± 11	163 ± 10	143 ± 15
Cu, mg/kg	16.9 ± 0.5	17.3 ± 1.0	18.0 ± 1.5	19.1 ± 1.6

[1] Treatments: Control (CON, no supplement); 2-methylbutyrate (MB, 12.3 mmol/kg DM); MB + isobutyrate (MB + IB, 7.7 and 12.6 mmol/kg DM, respectively); and MB + IB + isovalerate + valerate (ISO, 6.4, 7.3, 4.2, and 5.1 mmol/kg DM, respectively.

[2] Church & Dwight Co. Inc., Princeton, NJ.

[3] Zinpro Corporation (Eden Prairie, MN; 8.42, 8.51, 5.68, and 6.83% MB, IB, isovalerate, and valerate, respectively).

[4] Zinpro Corporation (Eden Prairie, MN; 25.6% IB).

[5] Zinpro Corporation (Eden Prairie, MN; 24.5% MB).

[6] Ag Processing Inc., Omaha, NE 68154.

[7] Premier Magnesia LLC, Overland Park, KS.

[8] The Mosaic Company, Tampa, FL.

[9] Zinpro Corporation (Eden Prairie, MN; Total Zn, 47,625 mg/kg; Zn-organic, 25,092 mg/kg; total Mn, 40,732 mg/kg; Mn-organic, 12,540 mg/kg; total Cu, 5,688 mg/kg; Cu-organic, 2,210 mg/kg; total Co, 630 mg/kg Co; Co-organic, 630 mg/kg; I, 804 mg/kg; Se, 203 mg/kg; folic acid, 1,767 mg/kg.

[10] At average DMI of 17.35 kg/d: vitamin (vit) A, 124,000; vit D, 22,000; and vit E, 565 IU/cow per day.

Results

Body Weight and Condition Score

Body weight did not differ by treatment but did tend (P = 0.10) to interact with week. These interactions will be explained through ADG responses over the entire post-covariate phase of the trial. Overall BW reported in Table 3.2 did not differ by treatment. There tended to be an interaction of treatment × parity (P =0.08) for BCS (Figure 3.1). Multiparous cows supplemented with ISO tended to have higher (P = 0.07) BCS than those supplemented with MB. Primiparous cows in the MB group were scored higher (P =0.04) than those fed CON. There was also a trend (P = 0.06) for an interaction of treatment × parity for ADG. Primiparous cows had a greater (P = 0.09) ADG (averaging 0.212 kg/d) compared to multiparous (0.106 kg/d). The parities behaved inversely (Figure 3.2). Multiparous cows in the CON group (-0.016 kg/d) had the lowest ADG within their parity, and ISO-supplemented cows (0.128 kg/d) had the highest. The CON multiparous cows tended to be different (P = 0.08) to ISO supplemented cattle, but there were no differences (P > 0.22) when comparing CON to MB+IB or MB. Whereas CON primiparous cows (0.337 kg/d) had the greatest ADG, the ISO-supplemented primiparous cows (0.083 kg/d) had the lowest. The CON primiparous cows were only lower (P = 0.05) than ISO supplemented cattle, but there were no differences (P > 0.17) when comparing CON to MB+IB or MB.

Milk Production and Milk Components

Milk, protein, fat, milk N, lactose yields were not affected by treatment ($P \geq 0.35$, Table 3.2) Fat yield for MB +IB was higher ($P < 0.03$) than MB and ISO treatments but not greater ($P = 0.39$) than CON. Treatment tended to interact ($P = 0.08$) with parity for fat percentage. There were no differences between treatments for primiparous cows; however, fat percentage was higher ($P < 0.03$, Figure 3.3) for MB + IB multiparous cows than multiparous cows receiving the other treatments. Treatment also interacted ($P = 0.05$) with parity for protein concentration. Multiparous cows fed the MB treatment had lower milk protein concentration ($P = 0.05$, Figure 3.3) than multiparous cows fed MB + IB but did not differ from ($P > 0.18$) from multiparous cows fed CON or ISO. There was no difference in milk NE_L secretion among treatment. Multiparous cows supplemented with MB tended to have higher ($P = 0.08$) SCC than CON cows. Primiparous cows supplemented with MB tended to have lower ($P = 0.08$) SCC than primiparous CON cows. Cows supplemented with MB + IB had greater ($P < 0.05$, Table 3.2) concentration of MUN than those fed MB or ISO. However, MB+IB did not differ ($P = 0.18$) from CON.

Intake and Efficiency

Main effects for biweekly efficiency measurements (i.e., milk N/N intake and milk NE_L/DMI) are reported in Table 3.2, but trends for treatment × time interactions ($P \leq 0.10$) are illustrated in Figure 3.4 and Figure 3.5, respectively. The only biweekly measurement with treatment differences for milk N/N intake was wk 7 and 8, when the ISO treatment

tended to be greater (P = 0.09) than the CON treatment. Milk N/N intake of cows supplemented with ISO were not different (P > 0.16) than cows supplemented with MB + IB or MB. Milk NE_L/DMI was greater (P = 0.03) for cows fed MB+IB than those fed the CON or MB treatments. When evaluating treatments over 2-wk intervals, only the first interval (wk 3 and 4) and third interval (wk 7 and 8) had differences between treatments. For the 1st interval, only MB + IB tended to be greater (P = 0.10) than CON, but no other treatments were different from each other (P > 0.20). For the third interval, MB + IB and were greater (P < 0.07, Figure 3.5) than CON, whereas the ISO treatment only tended to be greater (P = 0.07) than CON.

Table 3.2 Lactation performance of Jersey cows fed different combinations of branched-chain volatile fatty acids

	Treatment[1]					Significance[2]				
Item	CON	MB	MB+IB	ISO	SEM	TRT	WK	PR	TRT×WK	TRT×PR
Body Weight, kg [3]	409	405	408	410	7	0.60	< 0.01	< 0.01	0.10	0.69
ADG, kg/d[4]	0.160	0.202	0.136	0.138	0.061	0.86	—	0.09	—	0.06
BCS	2.68	2.71	2.71	2.73	0.15	0.69	0.03	< 0.01	0.82	0.08
Milk Production, kg/d										
Milk	25.9	25.5	25.7	25.4	0.2	0.35	< 0.01	< 0.01	0.94	0.96
Fat	1.15[ab]	1.13[b]	1.17[a]	1.13[b]	0.01	0.08	< 0.01	< 0.01	0.25	0.22
Protein	0.860	0.851	0.849	0.842	0.008	0.33	< 0.01	< 0.01	0.85	0.80
Milk N[5]	0.135	0.135	0.134	0.135	0.001	0.23	< 0.01	< 0.01	0.75	0.65
Lactose	1.22	1.22	1.20	1.20	0.001	0.39	< 0.01	< 0.01	0.81	0.48
Milk NE_L[6], Mcal/d	20.5	20.2	20.6	20.1	0.184	0.25	< 0.01	< 0.01	0.35	0.25
Milk Components										
Fat, % [3]	4.54[ab]	4.46[b]	4.63[a]	4.51[b]	0.05	0.04	< 0.01	0.06	0.44	0.08
Protein, %	3.36	3.33	3.37	3.36	0.02	0.26	< 0.01	< 0.01	0.99	0.05
Lactose, %	4.73	4.74	4.74	4.75	0.01	0.28	< 0.01	0.01	0.68	0.28
SCC, $\log_{10}$ cells/mL	4.70	4.70[a]	4.73	4.70	0.04	0.87	0.03	0.02	0.39	0.10
MUN, mg/dL [3]	13.9[ab]	13.7[b]	14.2[a]	13.8[b]	0.2	0.10	< 0.01	0.02	0.12	0.20
Intake and Efficiency[7]										
DMI, kg/d	17.6	17.3	17.3	17.7	0.7	0.95	0.39	< 0.01	0.33	0.44
N intake, kg/d	0.422	0.411	0.413	0.423	0.017	0.91	0.11	< 0.01	0.54	0.43
Milk N/N intake, kg/kg	0.330	0.327	0.347	0.342	0.014	0.66	< 0.01	0.77	0.10	0.21
Milk NE_L/DMI, Mcal/kg	1.19[ab]	1.18[b]	1.28[a]	1.23[ab]	0.05	0.34	0.35	0.67	0.05	0.21

[a-b]Mean values in the same row with different superscripts differ ($P \leq 0.10$) between treatments.

[1] Treatments: Control (CON, no supplement); 2-methylbutyrate (MB, 12.3 mmol/kg DM); MB + isobutyrate (MB + IB, 7.7 and 12.6 mmol/kg DM, respectively); and MB + IB + isovalerate + valerate (ISO, 6.4, 7.3, 4.2, and 5.1 mmol/kg DM, respectively.

[2] Probability of treatment effects: TRT = effects of different combination of branched-chain volatile fatty acids and valerate, WK = effect of week, PR = effect of parity, TRT×WK = interaction between supplementation and week, and TRT×PR = interaction between supplementation and parity.

[3] Interaction between week and parity ($P \leq 0.05$).

[4] ADG was averaged across week and not covariate-adjusted.

[5] Milk N was calculated by protein g/6.34 (NASEM, 2021).

[6] NE_L, Mcal/d = Milk Yield × [(0.0929 × Fat %) + (0.0563 × True Protein %) + (0.0395 × Lactose %)] (NASEM, 2021).

[7] Intake and efficiency measurements were analyzed after averaging biweekly

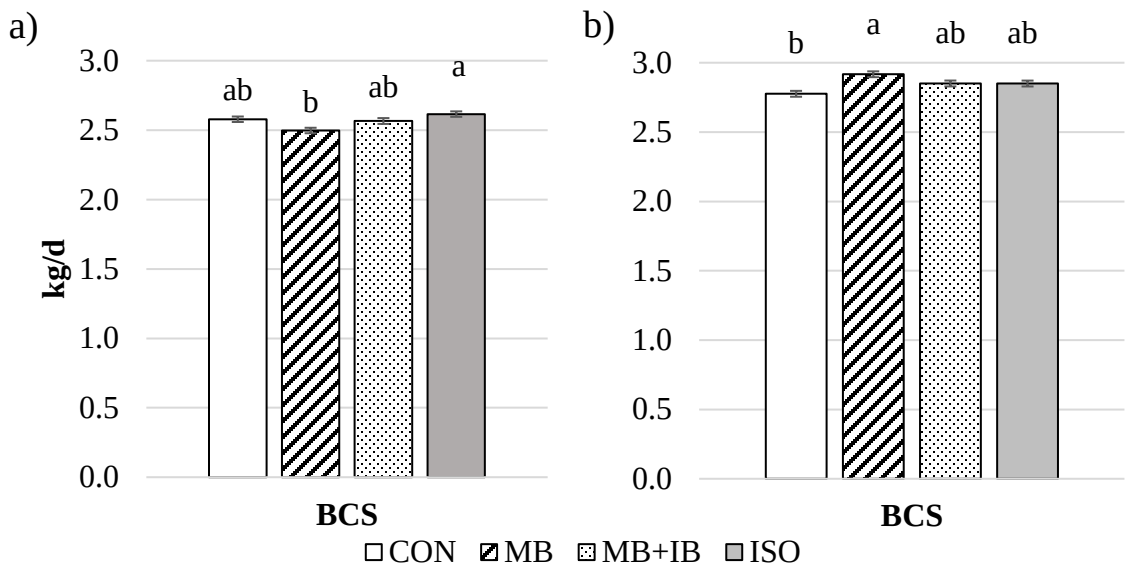

Figure 3.1. Body condition score (BCS) of multiparous (panel a) and primiparous (panel b) Jersey cows fed either control (CON, no supplement), 2-methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO).
The error bars are pooled SEM. Treatment × parity was $P \leq 0.08$. Means in the same graph with different superscripts tended to differ ($P \leq 0.10$).

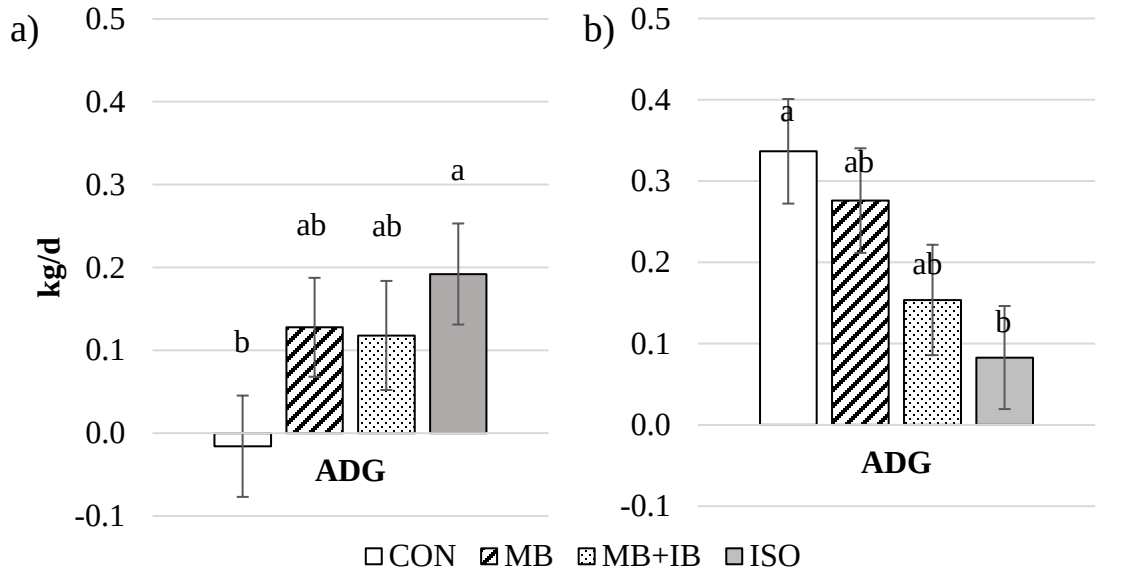

Figure 3.2. Average daily gain (ADG) of multiparous (panel a) and primiparous (panel b) Jersey cows fed either control (CON, no supplement), 2-methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO).
The error bars are pooled SEM. Treatment × parity was $P \leq 0.08$. Means in the same graph with different superscripts tended to differ ($P \leq 0.10$).

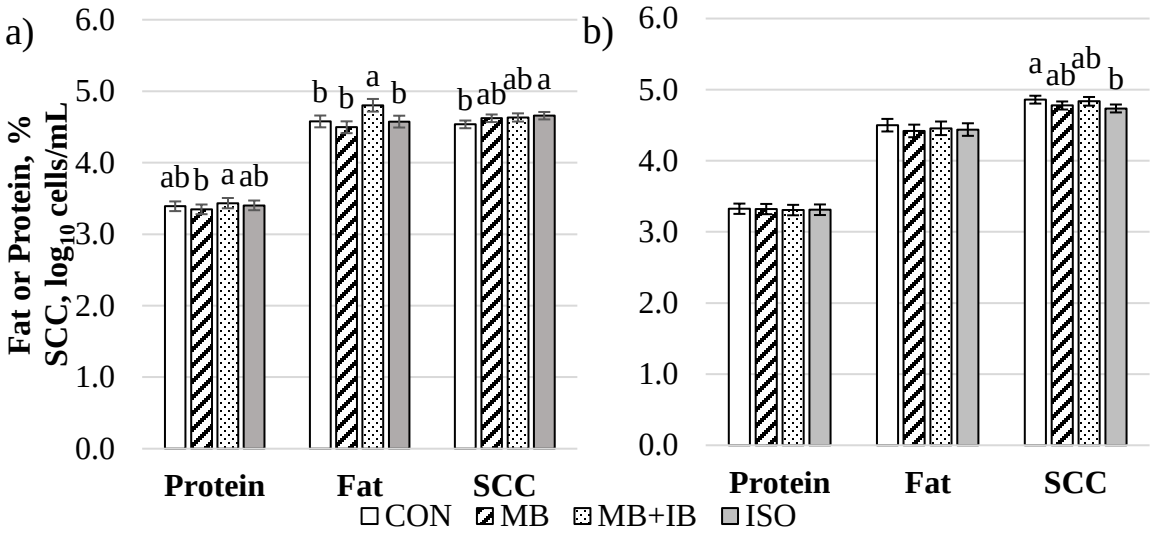

Figure 3.3. Milk component concentrations and SCC of multiparous (panel a) and primiparous (panel b) Jersey cows fed either control (CON, no supplement), 2-methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO).
The error bars are pooled SEM. Treatment × parity was $P \leq 0.08$. Means in the same graph with different superscripts tended to differ ($P \leq 0.10$).

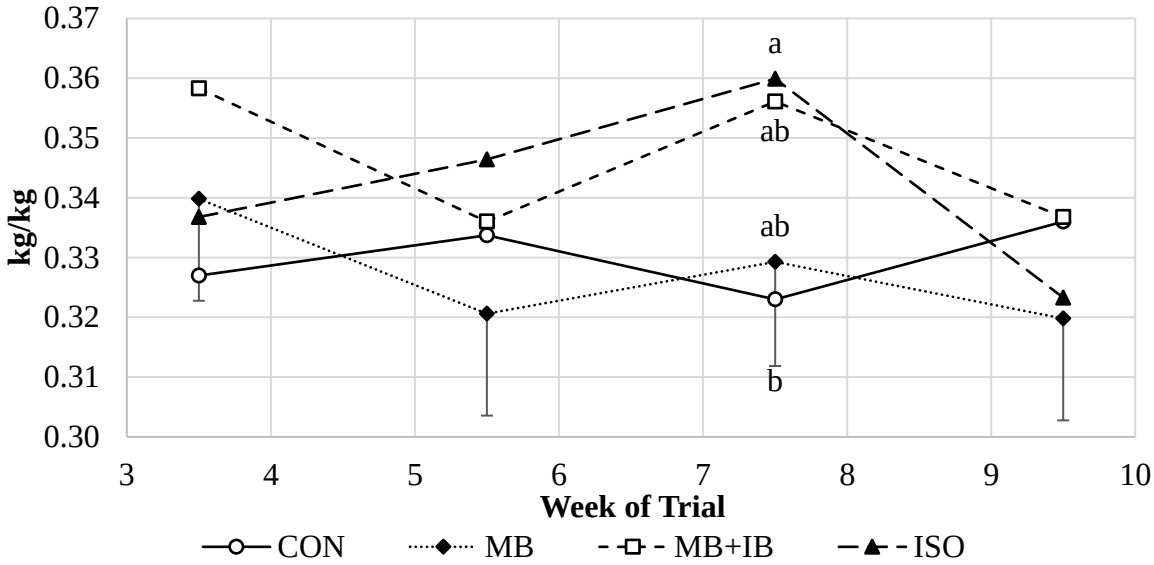

Figure 3.4. Milk nitrogen efficiency [milk N (kg/d)/DMI (kg/d)] for biweekly means from Jersey cows fed either control (CON, no supplement), 2-methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO).
The error bars are pooled SEM. Treatment × week was $P = 0.10$. Means at the same timepoint with different superscripts differ ($P \leq 0.10$).

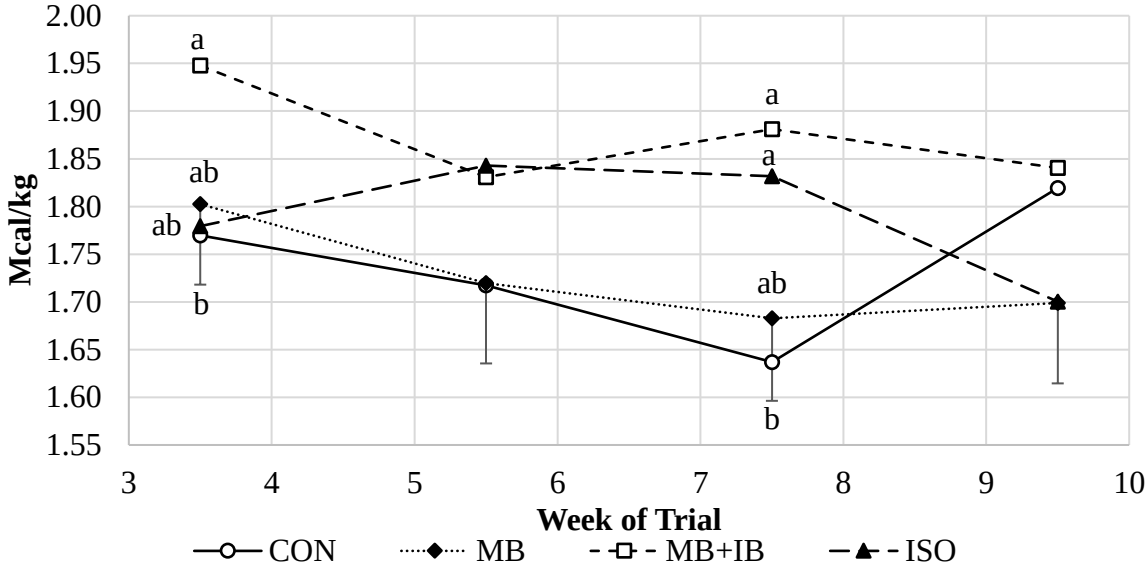

Figure 3.5. Milk energy efficiency [NE_L (Mcal/d)/DMI (kg/d)] for biweekly means from Jersey cows fed either control (CON, no supplement), 2-methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO).
The error bars are pooled SEM. Treatment × week was P = 0.05. Means at the same timepoint with different superscripts differ ($P \leq 0.10$).

Fatty Acid Profile

There were no treatment differences ($P = 0.39$) in FA yield (data not shown). There were few shifts in the individual FA profiles by treatment, so the partial sums of the FA profile are provided in Table 3.3, and the total FA profile is provided in Appendix A (Table A.1). There was a trend ($P = 0.09$) for a treatment difference for FA that were greater than 16 carbons in length ($\sum > 16$); this sum did not include BCFA to avoid double-counting for BCFA profiles and to emphasize de novo versus preformed FA in milk. There can be a small amount of BCFA produced de novo from BCVFA primers, particularly elongation (Prado et al., 2019), but there also is question regarding whether some BCFA might be

misidentified (Vlaeminck et al., 2015). The ∑ > 16 was lower ($P < 0.04$) for cows supplemented with MB and MB + IB than cows supplemented with ISO. However, MB and MB + IB did not differ from CON ($P > 0.18$). The decrease in ∑ > 16 was primarily a result of a treatment effect ($P = 0.04$) on 18:0 (Table A.1). Milk fat from cows supplemented with MB (10.4% of total FA) tended to be lower ($P < 0.08$) in 18:0 concentration compared to cows fed CON (11.6%) and ISO (12.2%) but did not differ ($P = 0.55$) from cows fed MB + IB (10.8%). Cows supplemented with MB + IB had lower ($P = 0.04$) 18:0 content than ISO-supplemented cows, but they did not differ ($P = 0.25$) from CON cows.

The total *anteiso* FA (∑ *anteiso* FA) concentration, shown in Table 3.3, experienced a trend for a 3-way interaction (TRT × WK × PR, $P = 0.08$). During wk 5, multiparous cows supplemented with MB had greater ($P < 0.04$) ∑ *anteiso* FA profile than those supplemented with MB + IB or ISO, but MB cows were not different (P = 0.25) than CON (Figure 3.6, panel a). On wk 9, multiparous cows fed ISO tended to have greater ($P < 0.10$) ∑ *anteiso* FA than multiparous cows supplemented with CON or IB + MB. Primiparous cows supplemented with MB during wk 5 tended to have greater ($P < 0.07$) ∑ *anteiso* FA than primiparous cows supplemented with CON or MB + IB (Figure 3.6, panel b); however, in wk 9, there were no treatment differences ($P > 0.44$). Results are mainly due to a difference in 15:0 *anteiso* FA content of milk FA (Table A.1). Cows fed MB had the highest 15:0 *anteiso* profile, which was greater ($P < 0.02$) than that for CON or MB + IB cows but not cows supplemented with ISO ($P = 0.13$). The CON, MB + IB, or ISO treatments did not differ ($P > 0.45$) in 15:0 *anteiso* content (Table A.1).

Table 3.3. Fatty acid profile of selected fatty acids and partial sums of milk produced of Jersey cows fed different combinations of branched-chain volatile fatty acids

	Treatment[1]					Significance[2]					
Fatty Acid (g/100g)	CON	MB	MB+IB	ISO	SEM	TRT	WK	PR	TRT×WK	TRT×PR	WK×PR
Σ < 16[3]	30.6	31.4	31.0	29.9	0.6	0.43	0.07	0.02	0.29	0.81	0.26
Σ 16[3]	33.9	34.9	34.7	33.3	0.6	0.30	< 0.01	0.35	0.87	0.19	0.06
Σ > 16[3]	31.6[ab]	29.8[b]	30.1[b]	32.8[a]	0.9	0.12	< 0.01	0.25	0.34	0.59	0.04
Σ18:1	16.9	16.4	16.3	17.5	0.5	0.07	< 0.01	0.30	0.32	0.59	0.06
ΣBCFA	1.12	1.16	1.10	1.12	0.02	0.42	< 0.01	0.07	0.54	0.37	< 0.01
Σ-*anteiso*[4]	0.493[b]	0.524[a]	0.477[b]	0.498[ab]	0.012	0.10	< 0.01	0.05	0.11	0.66	< 0.01
Σ-*iso* even	0.324	0.327	0.329	0.317	0.011	0.86	< 0.01	0.08	0.77	0.69	< 0.01
Σ-*iso* odd	0.307	0.314	0.298	0.306	0.008	0.57	< 0.01	0.62	0.70	0.34	< 0.01

[a-b] Mean values in the same row with different superscripts differ ($P \leq 0.10$) between treatments.

[1] Treatments: Control (CON, no supplement); 2-methylbutyrate (MB, 12.3 mmol/kg DM); MB + isobutyrate (MB + IB, 7.7 and 12.6 mmol/kg DM, respectively); and MB + IB + isovalerate + valerate (ISO, 6.4, 7.3, 4.2, and 5.1 mmol/kg DM, respectively.

[2] Probability of treatment effects: TRT = effects of different combination of branched-chain volatile fatty acids and valerate, WK = effect of week, PR = effect of parity, TRT×WK = interaction between supplementation and week, TRT×PR = interaction between supplementation and parity, and WK×PR = interaction between week and parity.

[3] Partial sums do not include BCFA.

[4] Interaction between supplementation, week, and parity ($P = 0.05$).

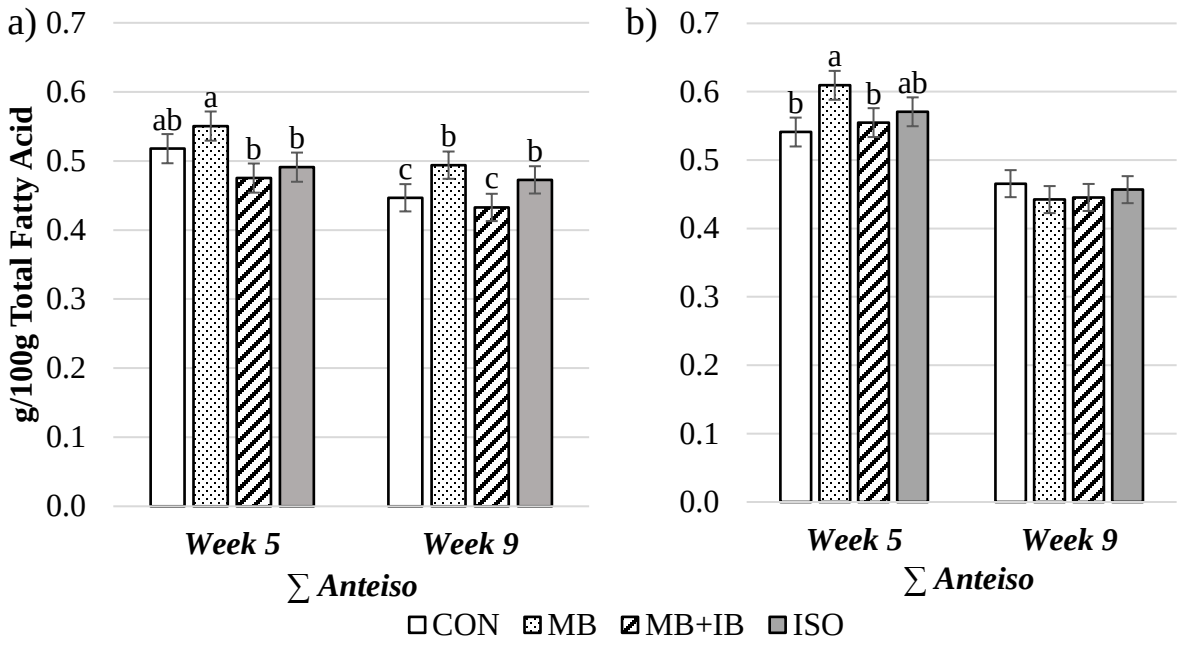

Figure 3.6. Anteiso fatty acid profile (g/100 g) in milk of multiparous (panel a) and primiparous (panel b) Jersey cows fed either control (CON, no supplement), 2-methylbutyrate (MB), MB + isobutyrate (MB + IB), or MB + IB + isovalerate + valerate (ISO).
The error bars are pooled SEM. Treatment × week × parity was P = 0.05. Means in the same graph with different superscripts tended to differ (P ≤ 0.10).

Discussion

In IsoPlus efficacy trials (Papas et al., 1984; Peirce-Sandner et al., 1985), cows started on trial at similar DIM, which contrasts with our trial where cows varying in DIM started all at one time. In these IsoPlus trials, the improved feed efficiency response decreased in the later weeks of the treatment period as all cows entered late lactation and were moved to a higher forage diet. All cows consumed the same diet in the current study, so fill would likely be less restricting, and improved NDF digestibility (and higher digestible energy status) should decrease DMI (Allen, 2020). Supplementation of BCVFA increased NDF degradation in vitro because many key cellulolytic bacteria require BCVFA

(Roman-Garcia et al., 2021a; Roman-Garcia et al., 2021b). Other responses in milk production were credited to BCVFA supplementation increasing celluloytic activity (Liu et al., 2014; Wang et al., 2015; Wang et al., 2018). Increased fiber degradation would increase the acetate: propionate ratio and increase acetate supply to the mammary gland, increasing de novo milk FA synthesis (Urrutia and Harvatine, 2017).

Compared to CON cows, cows fed MB + IB had numerically lower DMI while maintaining NE_L yield, which resulted in a tendency for improved feed efficiency in 2 of the measured time intervals (1^{st} and 3^{rd}), though the 2^{nd} approached a tendency ($P = 0.20$). Supplementation of only MB for *anteiso*-forming BCFA might have caused an imbalance in the rumen compared with concurrent provision of IB for *iso*-forming BCFA because bacteria often use the same enzymes for interlinking BCVFA metabolism, and some cellulolytics have a preference for IB or gain benefit of MB when there is adequate IB (Dehority et al., 1967). With MB supplementation at 15 g/d (~ 13.2 mmol/kg DM), Wang et al. (2018) noted improved feed conversion ratio [DMI (kg/d)/ADG (kg/d)] in Simmental steers. Bolus dosing only Ile (which pairs with MB) or Leu (which pairs with IV) depressed growth rate of ruminal bacteria in vitro, but Leu corrected the growth inhibition caused by Ile (Kajikawa et al., 2002; Kajikawa et al., 2005). The addition of Val did not suppress growth when supplemented alone and alleviated Ile growth inhibition in those studies. Therefore, assuming concentration-dependent transfer into bacterial cells, supplementation of either of the other two BCVFA might have been needed to maintain an optimal ratio of BCFA or BCAA intracellularly and might explain our lack of results for the MB treatment.

Increased production efficiency with MB + IB or ISO supplementation was not the result of losses in BW, as indicated by no overall BW differences among treatments. However, ADG was highest for ISO multiparous cows and the lowest for ISO-supplemented primiparous cows, when comparing to their respective CON treatments. The differences for ADG can be a result of energy partitioning among parity; multiparous cows are expected to lose more BW in early lactation and likely responsive to endocrine signals to replenish BW in mid and late lactation. In contrast, primiparous cows have flatter lactation curves and have not attained their mature BW during their entire first lactation (NASEM, 2021), which is supported by our BW measurements. Increasing the isobutyrate inclusion amount with multiparous cows increased ADG, which was reported by Liu et al. (2009b), but there was no difference in BW change in a following study in which multiparous cows were supplemented with all three BCVFA (Wang et al., 2019). However, the study of Liu et al. (2009b) had cows beginning the study at 148 DIM compared to 65 DIM in the study of Wang et al. (2019). Neither of these studies investigated BCVFA supplementation with primiparous cattle, which responded inversely compared to multiparous cows in our study. Thus, under our circumstances, supplementation of one or more BCVFA stimulated ADG with multiparous cows post peak milk production. These results demonstrate that the growth phase and energy status of the cows do influence the response to BCVFA supplementation, but improved production efficiency was consistent.

There were minor changes in the FA profile primarily with MB supplementation. When MB was supplemented alone, the increase in 15:0 *anteiso* concentration (the main FA in $\sum$ *anteiso*) could indicate that more MB was incorporated into microbial FA;

however, the MB treatment also had the highest daily dose of MB. Previous research with individual or mixed BCVFA supplementation reports that milk FA profile shift is limited. When supplementing with BCVFA and valerate, Copelin et al. (2021) did not report any shifts in BCFA in the milk individual FA profile.

The increase in 15:0 *anteiso* and ∑ *anteiso* FA with MB supplementation coincided with decreased 18:0 and decreased ∑ > 16 carbon FA. A decrease in FA greater than 16 carbons, because of increased *de novo* FA synthesis at the mammary gland, was consistent with previous studies using only multiparous cows (Liu et al., 2018a; Copelin et al., 2021). Increased acetate: propionate is a commonly reported benefit with BCVFA supplementation individually (Liu et al., 2009a; Liu et al., 2009b; Wang et al., 2012) or combined (Liu et al., 2018a; Liu et al., 2018b), but ruminal VFA concentrations were not measured in our current study. Copelin et al. (2021) noted that a blend similar to our ISO treatment numerically increased acetate: propionate and maintained milkfat production in a moderately milkfat-depressing diet. The current study was not designed to cause milkfat depression for which there was no evidence, as supported by the low 18:1 *trans*-10 concentration and lack of consistent detection of 18:2 *trans*-10, *cis*-12. There were no treatment differences for total 18:1 isomer concentration. The only isomer influenced by supplement was 18:1 *trans*-11, which was higher for multiparous cows fed CON but lower for primiparous cows fed CON.

Conclusion

Although our in vitro and in vivo work suggested the greatest need was for 2-methylbutyrate, the current production trial suggests that isobutyrate might need to be provided in addition to 2-methylbutyrate to improve lactation performance. Both DMI and milk yield were not significantly influenced by treatment, but milk fat yield and NE_L/DMI were increased with the MB + IB treatment. Both MB + IB and ISO supplementation has similar benefits for feed efficiency (NE_L/DMI), but the addition of IV and valerate did not increase efficiency compared to MB + IB. Additionally, the improvement in feed efficiency with MB + IB supplementation was not a result of change in BW, ADG, or BCS. Therefore, MB + IB appears the most efficacious, especially when Leu (producing IV) is relatively high in the diet due to the high inclusion rate of corn-based ingredients.

Chapter 4. Effects of branched chain volatile fatty acids supplementation on nutrient degradation, microbial functions, and prokaryotic profile in dual flow cultures varying forage and corn oil concentration

Introduction

The source of branched-chain volatile fatty acids (BCVFA) is branched-chain amino acids (BCAA) from RDP. Specifically, Val, Ile, and Leu are either transported for protein synthesis or deaminated or transaminated, and their keto acids are decarboxylated to the coenzyme A esters of isobutyrate, 2-methylbutyrate, and isovalerate, respectively. Early research identified rumen bacteria such as *Streptococcus bovis*, *Selenomonas ruminantium, Ruminobacter amylophilus*, *Megasphaera elsdenii*, *Prevotella ruminicola*, and *Butyrivibrio fibrisolvens,* that degraded BCAA to provide BCVFA that are used by mainly cellulolytic strains of *Fibrobacter* and *Ruminococcus,* which cannot degrade BCAA (Allison, 1978; Stackebrandt and Hippe, 1986; Walker et al., 2005). Some rumen bacteria can synthesize BCAA from central metabolites via the isopropylmalate pathway, but other bacteria such as *Ruminococcus flavefaciens, Prevotella ruminicola*, and *Megasphaera elsdenii* have a very low specific activity of isopropylmalate dehydrogenase compared to *E. coli* (Allison et al., 1966; Rogosa, 1971). Additionally, BCVFA can be

utilized for the synthesis of branched-chain lipids used in bacterial membrane structure. Thus, cross-feeding is critical for optimal growth of cellulolytics (Miura et al., 1980; Firkins, 2010) and the entire community (Morais and Mizrahi 2019).

Although increased NDF degradability and microbial N are commonly expected responses from BCVFA supplementation (Andries et al., 1987), research is needed to explain inconsistency among responses. For example, when BCVFA and valerate were supplemented in a diet designed to induce milk fat depression by Copelin et al. (2021), they did alleviate milk fat depression but did not influence NDF degradability or N metabolism. When 2-methylbutyrate was supplemented at 15 g/d to a 60:40 forage:concentrate or 40:60 forage:concentrate diet fed to Simmental steers (Wang et al., 2018), degradability of NDF increased, but the response was greater with 60% forage diet. Roman-Garcia et al. (2021b) noted BCVFA supplementation improved NDF degradability even when pH decreased, and solids passage rate was increased. However, Roman-Garcia et al. (2021c) detected no change in efficiency of microbial protein synthesis (EMPS), even with increased relative abundance of key cross-feeding partners *F. succinogenes* and *Treponema*, which both require BCVFA.

In comparison to the growth factor like BCVFA on cellulolytics, PUFA supplementation has bacteriostatic effects. Additional vegetable oil such as soybean or corn oil has been commonly used in research to suppress cellulolytic bacteria. Weld and Armentano (2017) have shown that 3% inclusion of corn oil can depress total tract NDF digestibility by 1.3%. The main fatty acids (FA) of corn oil are 52% linoleic, 30% oleic, and 13% palmitic (Gunstone, 1996). The inclusion of linoleic, or PUFA, is able to halt

growth of cellulolytic and some butyrate-producing bacteria (Maia et al., 2007). Whereas oleic and palmitic inclusion, supplemented at 1.5% DM, increased total tract NDF digestibility with a linear response to increasing oleic: palmitic ratio of FA in the supplement (de Souza et al., 2021). The mode of action for either suppression of NDF with linoleic FA or improvement with oleic and palmitic supplementation is not clearly known.

Our objective was to investigate the effect of BCVFA supplementation on EMPS under varying dietary conditions in dual flow continuous cultures. Supplementing BCVFA was hypothesized to increase cellulolytic activity and therefore NDF degradability. We hypothesized that increased forage:concentrate would increase the benefit of BCVFA because of more substrate but also that BCVFA would increase benefit from BCVFA supplementation in lower forage diets by increasing competition with amylolytics for BCVFA precursors for BCAA synthesis. Increased growth of cellulolytic bacteria was projected to increase the utilization of BCVFA into bacterial components, so less BCVFA will be measured in the outflow of fermenters. Finally, the addition of PUFA to the cultures was expected to challenge the rumen microbes because PUFA are bacteriostatic especially towards cellulolytic populations. We hypothesized that supplementation of PUFA would impede NDF digestibility, especially with lower forage, but suppression of NDF will be less when additional BCVFA are provided.

Material and Methods

Experimental Design and Treatments

The experiment was a 2 × 2 × 2 factorial arrangement of 8 treatments with 8 anaerobic dual-flow continuous culture vessels and 4 periods in a randomized incomplete block (n = 4). The vessels were randomly assigned to a treatment with either BCVFA supplementation (2.15 mmol/d of isobutyrate, 2.15 mmol/d isovalerate, and 2.15 mmol/d 2-methylbutyrate) or no additional BCVFA, high forage:concentrate diet (HF, 67:33) or low forage:concentrate diet (LF, 33:67), and either supplemental corn oil (CO, additional 3% of DM) or no additional fat supplement. Roman-Garcia et al. (2021b) previously justified 2 mmol/d dosages of each BCVFA. In our study, the dose was scaled to 2.15 mmol/d of each BCVFA to maintain the same ratio of BCVFA mmol/d to DMI. Additionally, because the BCVFA were prepared as acids, pH decreased by 0.06 units with BCVFA supplementation (Roman-Garcia et al., 2021b). In our study, the BCVFA doses were made with buffer, and drops of NaOH were added to bring the pH up to 6.8 (which is the pH before feeding in the vessels) just as was the control buffer. The diets (Table 4.1) were formulated with Spartan Ration Evaluator (v 3.0.3, Michigan State University, Department of Animal Science) to supply a 2nd lactation cow at 120 DIM. The forage:concentrate ratios were determined to maximize forage NDF (35.2% DM) in the HF diets and minimize starch (16.7% DM), and the LF diets were designed to minimize forage NDF (17.6% DM) and maximize starch (31.7% DM) relative to boundaries used in applied dairy nutrition. Total NDF, forage NDF, and starch were adjusted between HF and LF accordingly. All 4 diets were designed to be 15.0% CP by adding more soybean meal to

the low forage diets. The supplemental CO ranged from 50 to 60% linoleic and 20 to 30% oleic acids (Gunstone, 1996). Supplementation of CO at 3% DM would provide approximately a 1.5% DM dose of linoleic acid. This dose corresponded to the lowest dose, 1.5% - 4% DM, used in previous research on biohydrogenation using primarily soybean oil or corn oil, (Griinari et al., 1998; Rico and Harvatine, 2013).

Continuous Culture Operation

Two lactating Jersey cows were fed a standard lactation diet averaging 39% corn silage, 13.3% alfalfa hay, 20% ground corn, 9.9% wet brewer's grains, 7.7% soybean meal, 4.6% AminoPlus (AGP Ag Processing Inc.; a form of rumen-protected soybean meal), 0.9% Megalac (Church and Dwight), 0.9% molasses, and 3.4% of the remainder containing vitamins, minerals, and additives (no ionophores; data not shown). Cows were housed according to Institutional Animal Care and Use Committee standards at the Waterman Dairy in Columbus, Ohio. A total of 8 L of rumen contents were manually sampled through ruminal cannulas and squeezed through 2 layers of cheesecloth; the liquid was immediately transferred into prewarmed, insulated containers maintained at 39°C. All rumen fluid was pooled first and then inoculated into fermenters at 50% of fermenter working volume, with the remainder being filled with 39°C reduced buffer just prior to adding rumen fluid. The rumen fluid was added at 25% of the working volume in numerical order (fermenter 1 to fermenter 8) and then the final 25% was added in reverse (fermenter 8 to fermenter 1)

during every period. Treatments were randomized to fermenters. Clarified rumen fluid was added to the buffer for the first day of each period (Wenner et al., 2017).

Each period of the experiment was 12 d long, with 8 d of acclimation and 4 d of sampling. Buffer was continuously infused into the continuous cultures at a rate of 10% of vessel volume/h; the filtrate outflow was 5%/h, and the overflow passage was 5%/h. Thus, liquid and solids passage rates of 10 and 5%/h were maintained the same as in Roman-Garcia et al. (2021b). The buffer was made according to Weller and Pilgrim (1974), but 20 mg/dL of urea was added to maintain NH_3-N concentrations above 5 mg/dL at all times post-feeding. The temperatures of the vessels were maintained at 39°C, and the pH of the buffers being infused was adjusted, as described above for BCVFA, to remove differences among treatments. To minimize the diet effect on vessel pH, the HF diet buffer pH was balanced between 6.65 to 6.75 and the LF buffer pH between 6.75 to 6.85. All fermenters were checked hourly on d 1 and d 2 of each period to maintain the pH of all vessels between 6.8 prefeeding and 6.0 at the nadir post-feeding (see details below).

The cultures were fed 50 g DM/feeding and dosed their respective treatments (control buffer versus BCVFA treatment) 2 times a day at 12-h intervals. After background sampling (d 5) was completed, [2-methyl-^{13}C]-2-methylbutyrate, [2,3-$^{13}C_2$, 2-methyl-^{13}C]-isobutyrate, and [2,3,4-$^{13}C_3$, 3-methyl-^{13}C]-isovalerate (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) were bolus-dosed and replaced a portion of the respective unlabeled source to provide 5 mg/d of ^{13}C from each BCVFA while maintaining the same molar dose of total (labeled and unlabeled) BCVFA. Additionally, ^{15}N-enriched $(NH_4)_2SO_4$

was mixed into the buffer to continuously infuse 50 mg/d. All labeled sources were provided from d 6 through d 12.

Sample Collection and Processing

Data collection was between the morning and evening feedings only. On d 3 to 7, immediately before feeding at h 0 and every h thereafter, pH was measured to verify that buffers minimized any treatment effects. Reduction/oxidation potential (ORP) was also measured at the same time points. Immediately after feeding and at h 1, 3, 5, 7, 9, and 11, filtrate flow rate was measured and adjusted, as needed. At the same time points, the buffer pH was measured and adjusted with NaOH, as needed to maintain the different pH ranges between HF and LF. At h 1 and h 11, the buffer input rate was measured and calibrated to 10%/h. At h 12, prior to feeding, the total filtrate and overflow output were collected and weighed separately to measure filtrate, solids, and total outflow rates.

Effluents from the continuous cultures were collected on ice to prevent microbial activity outside of the dual-flow system. After combining filtrate and overflow after each 12-h feeding, duplicate 250-mL samples were subsampled from the total mixed outflow and pooled daily. One subsample was dosed with 8 mL of 5 *N* HCl to bring the pH down to 2 and stored at 4°C for 24 h. The next day, particle-associated bacteria were dissociated from feed so that our bacteria pellet included liquid- and particle-associated bacteria (Whitehouse et al., 1994). The collected pellet was used for bacterial N and C by elemental analysis and ^{15}N and ^{13}C enrichment by isotope-ratio mass spectrometry (Roman-Garcia et

al., 2021a). Supernatant during the processing of bacterial pellets was used for a colorimetric ammonia analysis (Chaney and Marbach, 1962). Another 20-mL sample of the supernatant was mixed with 5 mL of 50% TCA solution, and ammonia was diffused to filter disks prior to isotope-ratio mass spectrometry assay for ^{15}N enrichment (Hristov et al., 2001). The remaining 250-mL subsample was stored at -20 °C in a Whirl-pak® (Nasco, Fort Atkinson, WI) bag as a backup sample.

In the collection period (d 9 to 12), the continuous cultures were sealed for collection of gas data except when fed twice daily. A Micro-Oxymax Respirometer (Columbus Instruments Inc., Columbus, OH) continuously monitored 4 vessels for CH_4 and H_2 emission rate (mmol/min) and accumulation (mmol/d) during the collection period (n = 2). Measurements that did not require opening of the vessels, such as filtrate flow rate and buffer pH, were monitored at the same time points as before. Buffer input rates into the vessels were still monitored, but the cultures were clamped off at the buffer input port to prevent gas leaking. Samples for VFA analysis were taken from the filtrate effluent line at 0.5, 1, 2, 4, 8, and 12 h in relation to the time of dosage and feeding and were processed and analyzed for VFA by GLC as described by Roman-Garcia et al. (2021a). At 1, 2, 4, 8, and 12 h, a 20-mL sample from the liquid effluent line was taken and analyzed for aqueous H_2 [herein referred to as H_2(aq)] as described by Wenner et al. (2017).

. In addition to those samples collected and analyzed as described previously, a 5-mL sample of the total effluent from each 12-h interval was also processed, as described previously, to measure total VFA output from the vessel. Additional subsamples that were approximately 15% of the total effluent volume for each 12-h period were frozen, later

pooled over the collection period, and dried at 55°C. A subsample of the effluent was dried at 105°C overnight and then ashed at 550°C overnight to determine DM and OM. Residual NDF in the effluent was determined as described by Van Soest et al. (1991a) using amylase and sulfite. The effluent was also analyzed for ^{15}N and ^{13}C enrichments and N and C composition as previously described in Roman-Garcia et al. (2021c). Samples of diet ingredients were also analyzed for DM, OM, NDF, and N with the same methods. The feed samples were also analyzed by Cumberland Valley Analytical (Waynesboro, PA) for starch (Hall, 2009), ether extract (EE, AOAC, 2000; method 2003.05) and FA (Sukhija and Palmquist, 1988).

Additionally, duplicate 30-mL subsamples from the effluent were frozen at -80°C prior to pooling the 12-h samples within period. Genomic DNA was extracted using repeated bead beating plus column purification (Yu and Morrison, 2004) followed by sequencing with the Illumina MiSeq platform (Illumina Inc., San Diego, CA). Computational analysis was performed as described by Faulkner et al. (2017) except that amplicon sequence variants were recovered rather than operational taxonomic units, as described in Lee et al. (2021), and for Bray-Curtis dissimilarity, which was analyzed as described by Wenner et al. (2020).

After adjusting the ^{15}N enrichment for background (sampled before isotope dosing), the atom percentage excess (APE) was used in the following calculations. Bacterial N flow was calculated as total N flow × ^{15}N APE of effluent/^{15}N APE of bacteria. The bacterial N derived from NH_3-N was calculated as ^{15}N APE of bacteria/^{15}N APE of ammonia diffusions (Wenner et al., 2020). The NH_3-N flow was the product of NH_3-N

concentration in fluid and the total fluid effluent flow. The RUP was estimated as non-ammonia-non-bacterial N (NANBN) flow divided by the non-ammonia-N (NAN) flow. The RDP was the remaining NAN that was not recovered as NANBN. Net VFA production was the measured VFA production minus the amount of BCVFA dosed. Flow of C from effluent was the product of effluent percent C and effluent DM flow. The flow of bacterial C was calculated by multiplying the flow of bacterial N, by the ratio of C: N percent of the bacteria pellets. Recovery of ^{13}C was calculated by multiplying the ^{13}C APE of effluent or bacteria pellet by the respective C flow. Dose recovery was the µg ^{13}C recovered/ mg ^{13}C dosed.

Statistical Analysis

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

$$Y_{ijtlk} = \mu + D_i + C_j + B_t + (D \times C)_{ij} + (D \times B)_{it} + (C \times B)_{jt} + (D \times C \times B)_{ijt} + f_l + p_k + e_{ijtlk},$$

where Y_{ijtlk} is the dependent variable; μ is the overall population mean, D_i is the fixed effect of diet (i = HF or LF), C_j is the fixed CO supplementation (j = – CO or + CO), B_t is the fixed effect of BCVFA supplementation (t = – BCVFA or + BCVFA), $(D \times C)_{ij}$, $(D \times B)_{it}$, $(C \times B)_{jt}$, $(D \times C \times B)_{ijt}$ are the respective interactions of the main effects; f_l = is the random effect of lth vessel (l = 1 to 8); p_k is the random effect of kth period (k = 1 to 4); and e_{ijtlk} is the random error. Recovery of ^{13}C dose was analyzed with the same model, but an additional covariate was included LF had a higher computed RDP (calculated as explained

above) than HF, which would influence BCVFA precursor availability; therefore, BCVFA production (mmol/d) was included as the covariate to account for differing precursor availability.

Hourly data were analyzed using the same model but including the REPEATED (repeated measures) statement to assess the fixed effect of hour and all its interactions with the other main effects were included. The heterogeneous first-order autoregressive covariance structure (hourly pH and ORP) or the spatial power (for unequally spaced VFA, H_2(aq), and gas production) covariate structures were used based on the lowest Corrected Akaike Information Criterion. If there was an interaction with time, treatment means per hour were reported and contrasted at each time using the SLICE statement. Differences were declared at $P \leq 0.05$, and trends were $0.05 < P \leq 0.10$.

Table 4.1 Ingredients and nutrients of the diets varying in forage and corn oil supplementation.

Item	HF – CO[1]	HF + CO	LF – CO	LF + CO
Ingredient, % DM basis				
Alfalfa pellet	22.2	22.2	11.1	11.1
Orchard grass pellet	44.4	44.4	22.2	22.2
Concentrate pellet				
Corn grain, ground	22.9	15.9	44.2	44.2
Corn starch	—	5.00	—	—
Soybean meal, 48% CP	5.22	6.92	9.26	10.28
Soybean hulls	2.76	—	10.02	6.10
Fat, calcium soaps	1.40	1.40	1.40	1.40
Fat, corn oil	—	3.00	—	3.00
TM supplement[2]	0.50	0.50	0.50	0.50
Limestone	—	—	0.70	0.70
Dicalcium phosphate	0.20	0.20	0.20	0.20
Selenium, 200 mg/454 g	0.14	0.14	0.14	0.14
Magnesium oxide[3]	0.10	0.10	0.10	0.10
Vit E, 20,000 IU/g	0.07	0.07	0.07	0.07
Vit D, 3,000 IU/g	0.04	0.04	0.04	0.04
Vit A, 30,000 IU/g	0.02	0.02	0.02	0.02
Diet Composition, % DM basis				
DM, % as is basis	92.5	92.7	90.6	91.1
OM	89.9	89.7	92.2	92.3
NDF	38.8	38.2	28.3	28.1
Forage NDF	33.0	33.0	16.6	16.6
Starch	16.8	16.7	33.2	33.6
EE	3.07	5.12	4.06	5.88
FA	2.57	3.71	2.66	5.51
CP[4]	14.9	14.8	15.1	15.0

[1] Treatments are high (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil).

[2] Guaranteed analysis: Na, 36.6%; Cl, 56.4%; Zn, 3,500 mg/kg; Mn, 2,800 mg/kg; Fe, 1,750 mg/kg; Cu, 350 mg/kg; I, 70 mg/kg; and Co, 70 mg/kg.

[3] Premier Magnesia LLC (Conshohocken, PA).

[4] Predicted by Spartan Ration Evaluator (v 3.0.3, Michigan State University, Department of Animal Science) to be 64% of CP as RDP and 36% as RUP for HF – CO and HF + CO vs 62% RDP and 38% RUP for LF – CO and LF + CO based on the NRC (2001) model.

Results

pH and Reduction/Oxidation Potential

There were no main effect differences on pH ($P > 0.41$), which demonstrates the success of buffer adjustment with different forage:concentrate. There was a diet × CO

interaction (P = 0.09) for pH. In the HF – CO, HF + CO, LF – CO, and LF + CO treatments, mean pH was 6.37, 6.41, 6.41, and 6.40, respectively (data not shown); thus, these changes are deemed inconsequential to our objectives. There was a diet × hour interaction for pH, which is shown in Figure 4.1 (panel a). Only at h 0 (P = 0.02), 9 (P = 0.07), and 12 (P = 0.04) was the pH from LF treatments greater than that of HF by 0.03 units, which again was deemed inconsequential to our objectives. In contrast with pH, there were main effect differences for ORP (data not shown). Decreasing forage increased (P < 0.01) ORP (-318 mV for HF versus -310 mV for LF). When CO was supplemented, ORP decreased (P < 0.01) from -313 without CO compared to -315 with CO. There was also a diet × hour interaction (P = 0.05) for ORP (Figure 4.1, panel b). At all-time points, LF had a higher ORP than HF, but diet differences were less at h 0, 1, and 12 h post-feeding, which were timepoints near the 12-h feedings when degradation of new feed was less.

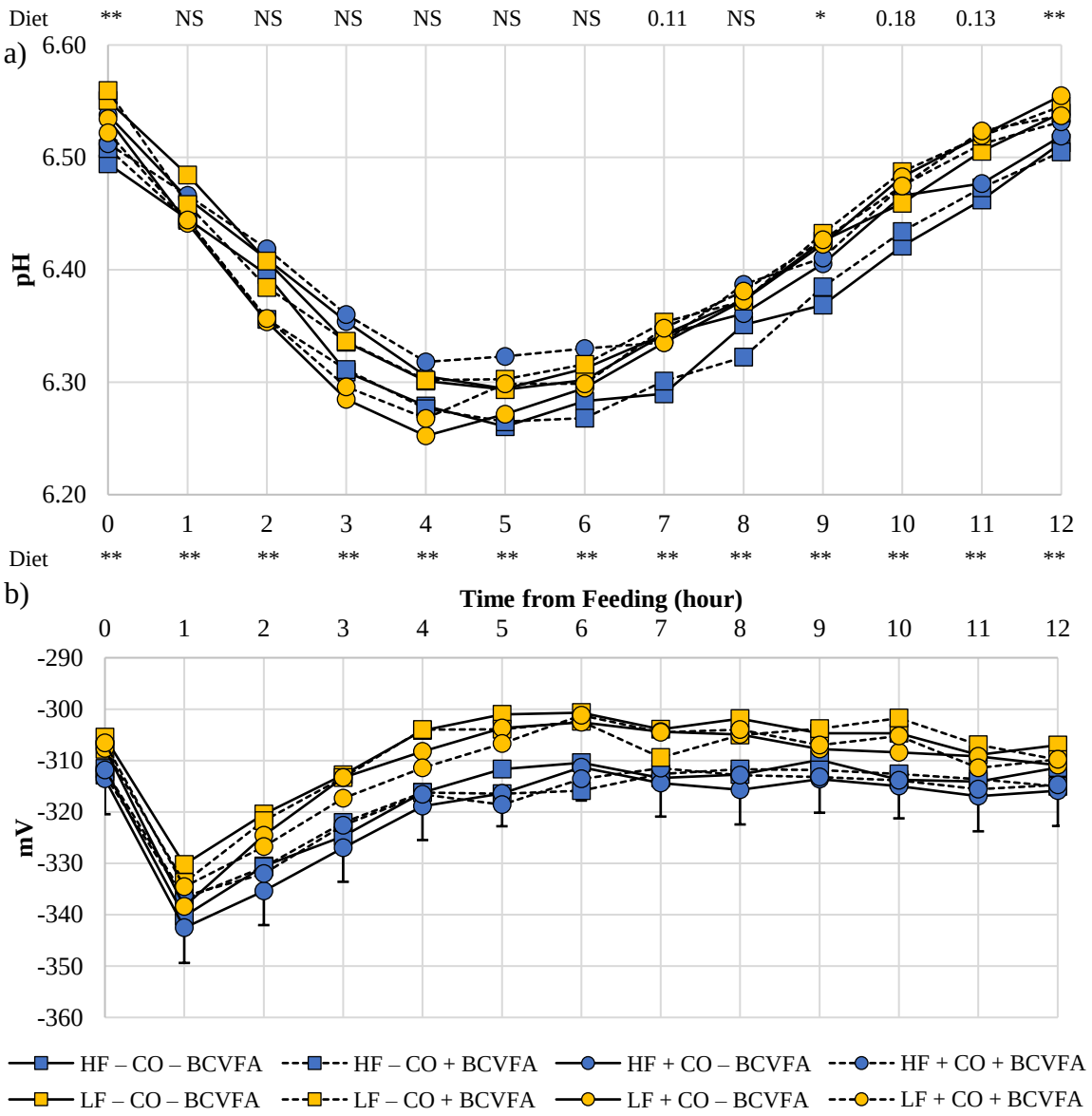

Figure 4.1. Fermenter pH (panel a) and oxidation/reduction potential (ORP, panel b) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA). Data are means from all 4 experimental periods taken on d 3 to 7 of the period. For pH, there was a diet × hour interaction ($P = 0.07$), but pH only differed by diet at h 0, 9, and 12 when pH was higher ($P < 0.08$, designated by * or $P < 0.05$ designated by **) with the main effect of LF compared to HF. For ORP, there was a diet × hour interaction ($P = 0.05$), but ORP was higher ($P < 0.05$; designated by **) for LF than HF at all time points. The SEM is pooled across treatment per time and shown for only 1 series per plot.

Nutrient Degradation and Flow of Nitrogen

The overflow and liquid passage rate ranged from 4.95 to 5.09 % volume/h and 9.81 to 9.93 % volume/h, respectively and were not affected by any main effect or interactions ($P > 0.20$). The LF diet had 6.7% greater true OM degradability than HF ($P < 0.01$, Table 4.1). Apparent starch degradability also tended to increase ($P = 0.10$) from 98.7% with HF to 99.2% with LF. There was a trend ($P = 0.10$) for the main effect of BCVFA supplementation to increase NDF degradability by 3.0 percentage units (i.e., 7.6%). There were no interactions with BCVFA supplementation; therefore, BCVFA benefited NDF degradability under all dietary conditions. There was a trend ($P = 0.07$) for an interaction of diet × CO, whereby CO supplementation in the LF diet increased ($P = 0.02$) NDF degradation by 8.2%, but CO supplementation with HF did not influence NDF degradation ($P = 0.98$).

The flow of total N experienced a 3-way interaction ($P = 0.07$, Table 4.2). The flow of NH_3-N only differed by diet, and NH_3-N flow decreased ($P < 0.01$) from 0.349 g/d with HF to 0.251 g/d with LF. The concentration of NH_3-N decreased ($P < 0.01$) from 8.38 mg/dL with HF to 6.15 mg/dL with LF. The flow of NAN also experienced a 3-way interaction ($P = 0.07$) resulting from the 3-way interaction ($P = 0.02$) from bacterial N, a component of NAN. Bacterial N flow increased with BCVFA supplementation, mainly due to an increase when supplemented in the HF – CO diet from 1.14 g/d (– BCVFA) to 1.38 g/d (+ BCVFA). Numerically bacterial N flow increased with BCVFA supplementation with LF – CO and LF + CO, but it numerically decreased with BCVFA supplementation with the HF + CO. Bacterial C flow also had a 3-way interaction ($P < 0.01$), which was

similar to bacterial N flow. Supplementation of BCVFA increased bacterial C flow with the HF – CO, LF – CO, and LF + CO diets compared to without BCVFA. However, bacterial C flow was decreased when BCVFA was supplemented with the HF + CO diet. The flow of NANBN decreased ($P < 0.01$) from 0.710 g/d with HF to 0.639 g/d with LF. Therefore, the 3-way interaction of total N flow is due to the 3-way interaction of bacterial N flow. The ratio of bacterial C: N decreased ($P < 0.01$) from 5.30 for HF to 5.07 with LF, but the bacterial N that originated from NH_3-N was not influenced by any main effects ($P > 0.14$). The RUP (i.e., NANBN / NAN × 100) decreased ($P < 0.01$) from 36.3% with HF to 33.5% with LF; RDP (100 – RUP) increased from 63.7% with HF to 66.5% with LF. Bacterial N/ kg truly degraded OM was greater ($P < 0.01$, Table 4.2) with the main effect of HF (25.1 g/kg) compared to LF (23.2 g/kg), and it also increased ($P = 0.03$) with the main effect of supplementing BCVFA from 23.4 to 24.9 g/kg of OM truly degraded. Supplementation of BCVFA increased ($P = 0.02$) bacterial N/g of truly degraded N by 0.05 g.

Table 4.2. Nutrient degradability, flow rates, and bacterial efficiency in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation.

	HF[1]				LF					Significance[2]				
	– CO		+ CO		– CO		+ CO		SEM					
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA	Diet × CO	Diet × CO × BCVFA
Degradability, %														
True OM	50.6	51.8	48.4	48.5	54.9	53.7	56.0	53.5	1.7	< 0.01	0.30	0.60	0.16	0.98
Apparent starch	98.3	98.9	98.7	99.0	99.3	99.1	99.3	98.9	0.4	0.10	0.75	0.72	0.53	0.61
NDF	39.2	41.0	37.8	42.2	36.1	39.5	43.1	45.4	3.3	0.58	0.08	0.10	0.08	0.40
Flow, g/d														
Total N	2.18	2.46	2.25	2.29	2.15	2.26	2.04	2.19	0.11	0.10	< 0.01	< 0.01	0.16	0.07
NH_3-N[3]	0.348	0.362	0.309	0.377	0.260	0.247	0.238	0.258	0.050	< 0.01	0.70	0.33	0.49	0.82
NAN[4]	1.83	2.09	1.94	1.91	1.89	2.01	1.80	1.93	0.088	0.36	0.15	0.01	0.16	0.07
Bacterial N[5]	1.14	1.38	1.23	1.19	1.26	1.31	1.21	1.30	0.05	0.26	0.19	0.01	0.06	0.02
Bacterial C[5]	6.03	7.50	6.36	6.26	6.26	6.59	6.10	6.77	0.27	0.50	0.16	< 0.01	0.08	< 0.01
NANBN[6]	0.691	0.712	0.718	0.720	0.631	0.701	0.592	0.632	0.059	< 0.01	0.45	0.19	0.15	0.92
RUP, % CP[7]	37.2	34.3	36.9	36.9	34.1	34.5	32.2	33.1	1.8	< 0.01	0.80	0.62	0.14	0.53
RDP, % CP[8]	62.8	65.7	63.1	63.1	65.9	65.5	67.8	66.9	1.8	< 0.01	0.80	0.62	0.14	0.53
Bacterial C: N	5.32	5.43	5.19	5.26	4.99	5.03	5.04	5.21	0.10	< 0.01	0.81	0.13	0.81	0.47
Bacterial N from NH_3-N	0.778	0.738	0.749	0.752	0.783	0.786	0.754	0.755	0.014	0.22	0.14	0.50	0.52	0.36
Efficiency of bacterial protein synthesis														
N/kg truly degraded OM	23.1	26.1	25.8	25.2	22.7	24.4	21.9	24.0	1.0	0.01	0.83	0.03	0.64	0.18
N/g truly degraded N	0.767	0.878	0.824	0.810	0.783	0.836	0.750	0.804	0.033	0.18	0.33	0.02	0.89	0.12

[1] Treatments are high (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] P values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] NH_3-N = ammonia nitrogen

[4] NAN (non-ammonia nitrogen) = total N - NH_3-N

[5] Interaction of CO and BCVFA supplementation has $0.02 \leq P \leq 0.08$

[6] NANBN (non-ammonia non-bacterial nitrogen) = total N - NH_3-N - bacterial N

[7] RUP = NANBN / NAN × 100

[8] RDP = 100 – RUP

Net production of Volatile Fatty Acids, Methane Emission, and Aqueous Hydrogen

For total VFA net production (Table 4.3), there was an interaction of diet × BCVFA ($P = 0.05$). There was a 2.87% decrease in net production in the HF when BCVFA was supplemented. However, when BCVFA was supplemented with LF, net production decreased by about 11.4%. Acetate and propionate production decreased by 11.4 and 5.53% ($P \leq 0.02$) with the main effect of BCVFA supplementation. Propionate production increased ($P < 0.01$) with LF compared to HF by 27.4%. Acetate: propionate decreased ($P < 0.01$) from 3.51 with HF to 2.78 with LF and decreased ($P < 0.09$) from 3.25 without CO to 3.03 with CO (all main effect differences). For butyrate, valerate, and caproate production, there were trends for interactions ($P \leq 0.10$) of diet × BCVFA. When BCVFA was supplemented with LF treatments, butyrate and valerate production decreased much more than when BCVFA was supplemented to HF. Caproate production's trend for a diet × BCVFA ($P = 0.10$) was the opposite; supplementation of BCVFA in the HF increased caproate production but decreased caproate in the LF treatments. The concentrations and molar proportions of the major and minor VFA in the vessels over time are shown in Figure C.1 to C.3 (Appendix C) and Figure D.1 to D.3 (Appendix D), respectively.

Net production of BCVFA is the total daily production minus their daily sum of both 12-h doses (Table 4.3). Isobutyrate ($P = 0.04$), 2-methylbutyrate ($P < 0.01$), and isovalerate ($P = 0.09$) had interactions with diet in which supplementation of BCVFA decreased the net production by twice or more in the LF diets compared with the decreased net production in the HF diets. Understandably, total BCVFA net production also had a similar interaction of diet with BCVFA ($P = 0.01$). The concentrations and molar

proportions of individual BCVFA and total BCVFA over time are shown in Figure C.4 to C.5 (Appendix C) and Figure D.4 to D.5 (Appendix D), respectively.

Compared with the main effect of HF, LF increased ($P < 0.01$) H_2 emission (Table 4.3). This increase is supported by H_2(aq) concentrations (Figure B.1, Appendix B) and H_2 production rate expressed over time (Figure B.2) because of diet × time interactions ($P < 0.01$). The LF H_2(aq) concentrations and H_2 production rates were only greater than HF during the first 4 h after feeding after which treatments converged as remaining degradable substrate declined. Methane production (Table 4.3) did not differ by diet ($P = 0.48$), but there was a trend for a diet × BCVFA ($P = 0.10$) and CO × BCVFA ($P = 0.09$) interaction. With BCVFA supplementation, CH_4 production numerically increased in the HF diet from 91.9 mmol/d to 102 mmol/d, whereas with LF, the supplementation of BCVFA decreased CH_4 production from 114 mmol/d to 82.6 mmol/d. Whereas BCVFA supplementation without CO decreased CH_4 production from 111 mmol/d to 87.9 mmol/d, but did not affect CH_4 production with CO. Methane production rate (Figure B.2) also had a diet × time, and diet × CO × time interaction ($P = 0.02$). The differences by diet were within h 3 to 7 because CH_4 production rate peaked later for HF than LF and decreased at a slower rate than that for LF.

Isotope Recovery

The ^{13}C from dosed BCVFA, as recovered in the total effluent (including bacteria, recycled products of microbial metabolism not retained in bacteria, and unmetabolized

dose) and in collected bacteria pellets after both were converted to daily outflows through total collection of effluent and using the ^{15}N marker approach to estimate bacterial N outflow (Table 4.3). There was no difference ($P \geq 0.14$) in the recovery of dosed ^{13}C from BCVFA from the total effluent flow, but recovery in bacterial C outflow decrease (P = 0.03) from 144 µg/ mg with HF to 98.9 µg/ mg with LF. Recoveries of ^{13}C in individual BCAA and branched-chain lipids in bacteria will be reported in Chapter 5 and Chapter 6.

Table 4.3. Net production of VFA, gas production, and dose recovery in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation.

	HF[1]				LF					Significance[2]			
	– CO		+ CO		– CO		+ CO		SEM				
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA	Diet × BCVFA
Net Production, mmol/d													
Total VFA	379	379	388	367	430	384	446	392	18	< 0.01	0.60	< 0.01	0.05
Acetate	249	254	254	241	252	235	258	225	11	0.25	0.65	0.02	0.11
Propionate	73.4	70.6	74.7	70.5	94.3	83.0	101.0	90.0	5.5	< 0.01	0.18	0.01	0.17
Acetate: Propionate	3.45	3.66	3.46	3.46	2.75	3.13	2.62	2.60	0.20	< 0.01	0.09	0.27	0.77
Butyrate	41.1	41.2	43.9	42.3	64.0	55.5	66.5	59.0	3.6	< 0.01	0.19	0.02	0.06
Valerate	5.80	5.85	6.05	5.88	7.77	6.73	7.95	6.66	0.42	< 0.01	0.66	0.01	0.01
Caproate	2.23	2.61	2.23	2.31	4.49	4.05	4.58	3.60	0.44	< 0.01	0.56	0.39	0.10
Isobutyrate	2.63	2.62	2.85	2.41	3.24	2.55	3.36	2.55	0.25	0.02	0.79	< 0.01	0.04
2-Methylbutyrate	2.28	2.08	2.46	1.91	3.86	2.71	3.85	2.56	0.32	< 0.01	0.82	< 0.01	0.01
Isovalerate	2.28	2.28	2.55	2.14	3.46	2.79	3.63	2.75	0.21	< 0.01	0.63	< 0.01	0.03
Total BCVFA	7.19	6.98	7.88	6.46	10.5	8.04	10.9	7.87	0.77	< 0.01	0.84	< 0.01	0.01
Gas emission, mmol/d													
H_2	0.202	0.153	0.209	0.099	0.391	0.468	0.498	0.469	0.086	< 0.01	0.73	0.55	0.40
CH_4[3]	99.7	102	81.3	107	124	75.8	108	86.8	28.8	0.48	0.37	0.21	0.10
Total ^{13}C recovery in C flow, µg/mg of ^{13}C dosed[4]													
Total effluent	—	943	—	880	—	800	—	806	41	0.15	0.55	—	—
Bacteria pellet	—	156	—	133	—	95.1	—	103	7	0.03	0.35	—	—

[1] Treatments are high (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] *P* values for main effect means for diet (HF vs LF), CO, BCVFA or interaction; interactions not shown have $P > 0.10$.

[3] Interaction of CO and BCVFA supplementation has $P \leq 0.09$

[4] ^{13}C was only provided with the BCVFA treatment (5 mg/d ^{13}C of each isobutyrate, 2-methylbutyrate, and isovalerate); therefore, there is no dose recovery in – BCVFA treatments.

Prokaryotic Profile Changes

Diet overwhelmingly influenced α diversity metrics (Table 4.4). The main effect of LF decreased ($P < 0.04$) most of the metrics compared with the main effect of HF except for Good's Coverage, which increased ($P = 0.01$) from 0.996 with HF to 0.997 with LF. Only Simpson's Index was unaffected by treatment. For β diversity, which was compared using Bray-Curtis dissimiliary (Figure 4.2), only diet ($P < 0.01$) and period ($P < 0.01$) of the study supported a major shifts.

The relative abundance of individual ASVs (taxonomically equivalent to species) were combined into genus and phylum ranks (Table 4.4). Not all ASV can be annotated or met the criterion (at least 0.5 % relative abundance for an observation) by our approach. However, when discernible, if sequences from only 1 ASV made up the entire genus or if a single genus made up the entire phylum, the only the lowest taxonomic rank was shown to avoid duplication. Bacteroidetes, the most abundant phylum, decreased ($P < 0.01$) from 56.3% with HF to 41.9% with LF. The uncultured genus *BF311*, within the Bacteroidetes phylum, decreased ($P < 0.01$) with decreasing forage content. Additionally, supplementation of BCVFA to HF also decreased ($P = 0.06$, diet × BCVFA) the relative abundance of *BF311*. The major genus within this phylum is *Prevotella*, which was not affected by diet ($P = 0.28$), although these was a shift among the species resolved within *Prevotella*. The characterized *P. ruminicola* and another uncharacterized ASV increased ($P \leq 0.04$) with LF, whereas the other predominant uncharacterized ASV decreased in relative sequence abundance. One unknown genus within the Bacteroidetes phylum did not change ($P = 0.98$) by diet. However, supplementation of CO to HF decreased ($P = 0.06$,

diet × BCVFA) the relative abundance of *BF311*, whereas CO supplementation to the LF diet had oppostive effect on this uncultured genus. Another uncharacterized genera, CF231 and YRC22, decreased ($P < 0.01$) for LF versus HF.

The phylum Fibrobacteres also decreased ($P < 0.01$) from 0.31% with HF to 0.14% with LF, and *Fibrobacter succinogenes* was the only species represented in this phylum. Diet did not influence Firmicutes relative abundance, but this is a diverse phylum that contains the order Clostridiales and many genera within the order that did shift by diet. *Butyrivibrio* decreased ($P < 0.01$) with decreasing forage and decreased ($P = 0.10$) with supplemented CO. A diet × CO interaction ($P = 0.10$) was detected for the genus *Ruminococcus*, with its relative abundance being lower with HF – CO than HF + CO but decreasing with LF + CO. This was mainly due to a shift with *R. albus*, which also had a diet × CO interaction ($P = 0.03$) explained by increased relative abundance with CO supplementation in the HF diet but decreased relative abundance with CO supplementation in the LF diet. Relative abundance of *R. bromii* and *R. flavefaciens* had opposite diet × BCVFA interactions ($P < 0.10$). The relative sequence abundance of *R. bromii* increased with BCVFA supplementation in the HF diet but decreased with BCVFA supplementation with LF, whereas *R. flavefaciens* decreased with BCVFA supplementation in the HF diet but increased with BCVFA supplementation with LF. There were few main effects of CO on relative abundance. However, *Anaerovibrio* increased with CO supplementation but increased with LF compared to HF.

The phylum Proteobacteria increased ($P < 0.01$) in relative sequence abundance from 5.79% with HF to 18.7% with LF, much of this shift occurred in an unknown genus

in the Succinivibrionaceae family that increased from 4.24% with HF to 10.1% with LF. The relative abundance of *Ruminobacter* increased ($P < 0.01$) with additional CO but did not change with forage ($P = 0.26$). *Succinimonas*, which was represented only by *S. amylolytica*, increased ($P < 0.01$) with supplemented CO and increased ($P = 0.01$) with decreasing forage. The genus *Succinivibrio* also increased ($P = 0.01$) with decreasing forage. The candidate phylum SR1 decreased ($P < 0.01$) with decreasing forage. The abundances of the phyla Spirochaetes, TM7, or Verrucomicrobia did not change by diet. Within Spirochaetes, the relative sequence abundance of *Treponema* decreased ($P = 0.05$) with CO, but no genera or species abundance within Spirochaetes, TM7, or Verrucomicrobia were influenced by any dietary conditions ($P > 0.18$). The phylum Tenericutes abundance decreased ($P < 0.01$) with decreasing forage. The unknown phylum had a diet × CO interaction ($P = 0.06$) in which supplemental CO decreased the abundance moderately with LF, but more so when CO was supplemented with the HF diet.

Table 4.4. Alpha diversity metrics and prokaryotic profile in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation.

	HF[1]				LF				SEM	Significance[2]		
	– CO		+ CO		– CO		+ CO					
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA
Alpha Diversity												
Observed ASV	1402	1411	1393	1417	980	1127	1006	1100	96	< 0.01	0.99	0.28
Chao1	1487	1484	1475	1506	1039	1176	1074	1161	110	< 0.01	0.92	0.38
Evenness	0.780	0.779	0.803	0.789	0.735	0.746	0.728	0.730	0.034	0.04	0.92	0.98
Faith's Phylogenetic Diversity	65.6	66.4	65.2	66.5	54.5	58.0	54.3	57.0	2.5	< 0.01	0.84	0.23
Shannon's Index	8.15	8.15	8.38	8.27	7.29	7.57	7.24	7.36	0.39	0.01	0.93	0.80
Simpson's Index	0.972	0.965	0.986	0.967	0.959	0.971	0.963	0.964	0.017	0.49	0.78	0.80
Good's Coverage	0.996	0.996	0.996	0.996	0.997	0.997	0.997	0.997	0.001	0.01	0.66	0.68
Relative Abundance, %[3]												
Euryarchaeota	0.0854	0.127	0.0843	0.0875	0.0642	0.0761	0.0534	0.0550	0.0282	0.10	0.37	0.47
Methanobrevibacter	0.0733	0.109	0.0744	0.0732	0.0481	0.0706	0.0501	0.0522	0.0251	0.14	0.48	0.41
Bacteroidetes	58.2	57.1	55.6	54.2	43.1	42.9	41.4	40.1	3.2	< 0.01	0.18	0.58
BF311[4]	0.695	0.603	0.756	0.546	0.333	0.267	0.210	0.279	0.072	< 0.01	0.50	0.07
Prevotella	16.8	15.0	18.1	17.6	15.2	16.2	15.5	16.4	1.6	0.28	0.28	0.92
ruminicola	3.87	2.88	3.51	4.02	4.56	4.28	4.50	5.42	0.88	0.04	0.37	0.94
unknown ASV[5]	0.915	0.676	0.995	0.928	1.11	1.39	1.08	1.06	0.178	0.01	0.94	0.91
unknown ASV	12.1	11.5	13.6	12.6	9.5	10.5	9.9	9.9	1.0	< 0.01	0.39	0.86
Unknown Genus[5]	2.16	1.52	2.71	2.57	2.20	2.56	1.90	2.33	0.58	0.98	0.39	0.98
CF231	0.584	0.587	0.476	0.577	0.407	0.371	0.284	0.416	0.079	< 0.01	0.34	0.32
YRC22	1.89	2.16	2.21	2.20	1.28	1.23	1.24	1.18	0.28	< 0.01	0.67	0.80
unknown Genus	0.367	0.129	0.163	0.0971	0.179	0.119	0.136	0.181	0.0745	0.45	0.25	0.10
unknown Genus	1.03	1.20	1.02	1.05	0.767	0.772	0.671	0.611	0.141	< 0.01	0.22	0.65
Fibrobacteres												
Fibrobacter succinogenes[6]	0.300	0.401	0.269	0.274	0.147	0.169	0.135	0.118	0.059	< 0.01	0.19	0.51
Firmicutes	27.8	28.7	29.3	29.1	29.2	31.1	29.7	31.8	5.4	0.44	0.73	0.85
Clostridium	1.04	1.22	1.18	1.22	0.91	0.92	0.99	0.81	0.40	< 0.01	0.13	0.71
Anaerostipes	0.127	0.093	0.129	0.150	0.207	0.229	0.283	0.329	0.066	< 0.01	0.10	0.63
Butyrivibrio	3.15	3.47	2.69	2.59	2.15	2.35	1.81	1.98	0.58	0.01	0.26	0.76
Clostridium	0.0257	0.0138	0.03	0.0323	0.0158	0.0193	0.025	0.0271	0.0086	0.55	0.07	0.19
Coprococcus	0.747	0.707	0.898	0.794	0.689	0.826	0.991	0.852	0.132	0.37	0.03	0.54
Lachnospira pectinoschiza[5]	0.153	0.160	0.183	0.206	0.0962	0.879	0.0828	0.939	0.0263	< 0.01	0.17	0.50

continued

Table 4.4 continued

	HF[1]				LF				SEM	Significance[2]		
	– CO		+ CO		– CO		+ CO					
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA
Moryella	0.0467	0.0698	0.0527	0.0602	0.0259	0.0262	0.0397	0.0362	0.0131	0.01	0.58	0.46
Pseudobutyrivibrio	1.61	1.96	1.72	1.72	1.70	1.89	1.88	2.05	0.34	0.51	0.79	0.38
Shuttleworthia	0.146	0.218	0.168	0.199	0.205	0.205	0.250	0.286	0.050	0.05	0.23	0.19
Unknown Genus	2.47	2.31	3.72	3.19	7.71	6.95	8.71	9.68	2.77	< 0.01	0.29	0.93
Unknown Genus	1.41	1.40	1.46	1.27	0.80	0.88	0.68	0.80	0.15	< 0.01	0.43	> 0.99
Oscillospira	0.678	0.744	0.818	0.782	1.646	1.191	1.343	1.618	0.334	0.01	0.75	0.87
Ruminococcus[5]	1.71	1.77	1.97	1.91	1.80	1.96	1.57	1.71	0.30	0.53	0.86	0.54
albus[5]	0.122	0.109	0.143	0.122	0.121	0.136	0.0947	0.0944	0.0355	0.25	0.44	0.68
bromii[4]	0.0140	0.0513	0.0533	0.0411	0.165	0.0094	0.0773	0.0286	0.0638	0.37	0.77	0.19
flavefaciens[4]	0.352	0.286	0.358	0.304	0.109	0.158	0.124	0.161	0.070	< 0.01	0.68	0.74
unknown ASV	1.10	1.20	1.22	1.28	1.31	1.56	1.21	1.35	0.19	0.15	0.81	0.22
Unknown Genus	1.07	1.02	1.13	0.907	0.685	1.06	0.598	0.555	0.167	0.01	0.14	0.88
Unknown Genus	2.52	2.76	2.68	2.79	1.77	1.87	1.58	1.74	0.33	< 0.01	0.88	0.46
Anaerovibrio	0.0846	0.104	0.135	0.162	0.190	0.185	0.211	0.211	0.0254	< 0.01	0.02	0.47
Selenomonas ruminantium[4]	0.0171	0.0146	0.0369	0.0497	0.0522	0.0519	0.0854	0.108	0.0314	0.57	0.22	0.25
Succiniclasticum	1.75	1.56	1.47	1.37	1.56	1.83	1.62	1.55	0.18	0.34	0.11	0.82
Unknown Genus	0.416	0.491	0.498	0.472	0.313	0.410	0.312	0.312	0.066	< 0.01	0.78	0.28
Proteobacteria	4.67	4.01	6.74	7.73	18.2	17.4	20.9	18.4	5.09	< 0.01	0.42	0.80
Unknown Genus	0.246	0.335	0.453	0.432	0.257	0.092	0.164	0.202	0.161	0.05	0.38	0.87
Vitreoscilla	0.254	0.387	0.414	0.544	0.261	0.346	0.267	0.181	0.239	0.22	0.71	0.54
Ruminobacter	0.0724	0.112	0.177	0.279	0.123	0.117	0.274	0.278	0.0684	0.26	< 0.01	0.30
Succinimonas amylolytica	0.0171	0.0146	0.0369	0.0497	0.0522	0.0519	0.0854	0.108	0.0314	0.01	0.02	0.56
Succinivibrio	1.21	1.00	1.53	1.31	2.85	5.08	4.18	3.57	1.45	0.01	0.91	0.76
Unknown Genus	0.80	0.25	1.34	1.85	10.9	7.85	11.4	10.1	3.84	< 0.01	0.54	0.59
Unknown Genus	0.689	0.456	1.18	1.62	2.76	3.01	3.62	3.09	1.21	< 0.01	0.32	0.98
Acinetobacter	0.334	0.449	0.524	0.506	0.371	0.231	0.326	0.192	0.169	0.08	0.68	0.64
unknown ASV[4]	0.178	0.416	0.374	0.478	0.344	0.221	0.286	0.166	0.148	0.18	0.64	0.75
unknown ASV	0.146	0.009	0.108	0.015	0.009	0.007	0.020	0.019	0.055	0.15	0.95	0.14
SR1	0.385	0.316	0.396	0.370	0.206	0.184	0.154	0.151	0.091	< 0.01	0.91	0.54
Spirochaetes	4.81	5.71	3.94	4.48	6.43	5.31	5.03	6.53	2.17	0.39	0.65	0.72
Sphaerochaeta	2.88	3.17	2.24	2.44	4.09	2.23	3.58	4.76	1.61	0.32	0.87	0.96
Treponema	1.81	2.33	1.51	1.76	2.17	2.91	1.13	1.32	0.72	0.94	0.05	0.31
TM7	0.541	0.342	0.477	0.479	0.585	0.595	0.534	0.520	0.141	0.20	0.86	0.51
Tenericutes	1.88	1.91	1.83	2.03	1.11	1.29	1.21	1.24	0.21	< 0.01	0.84	0.48
Anaeroplasma	0.959	0.911	0.801	0.600	0.328	0.350	0.215	0.236	0.174	< 0.01	0.13	0.64

continued

Table 4.4 continued

	HF[1]				LF				SEM	Significance[2]		
	– CO		+ CO		– CO		+ CO					
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA
Verrucomicrobia	0.307	0.328	0.423	0.381	0.354	0.292	0.344	0.360	0.096	0.62	0.20	0.70
Unknown Phylum[5]	0.547	0.555	0.341	0.313	0.199	0.276	0.198	0.242	0.074	< 0.01	0.03	0.64

[1] Treatments are high (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] P values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] Phyla are followed by genera and by species when classification is possible; if either a phylum or genus contains a single species, only the relative sequence abundance at the species rank is shown.

[4] Interaction of diet and BCVFA supplementation has $0.06 \leq P \leq 0.07$.

[5] Interaction of diet and CO supplementation has $0.03 \leq P \leq 0.09$.

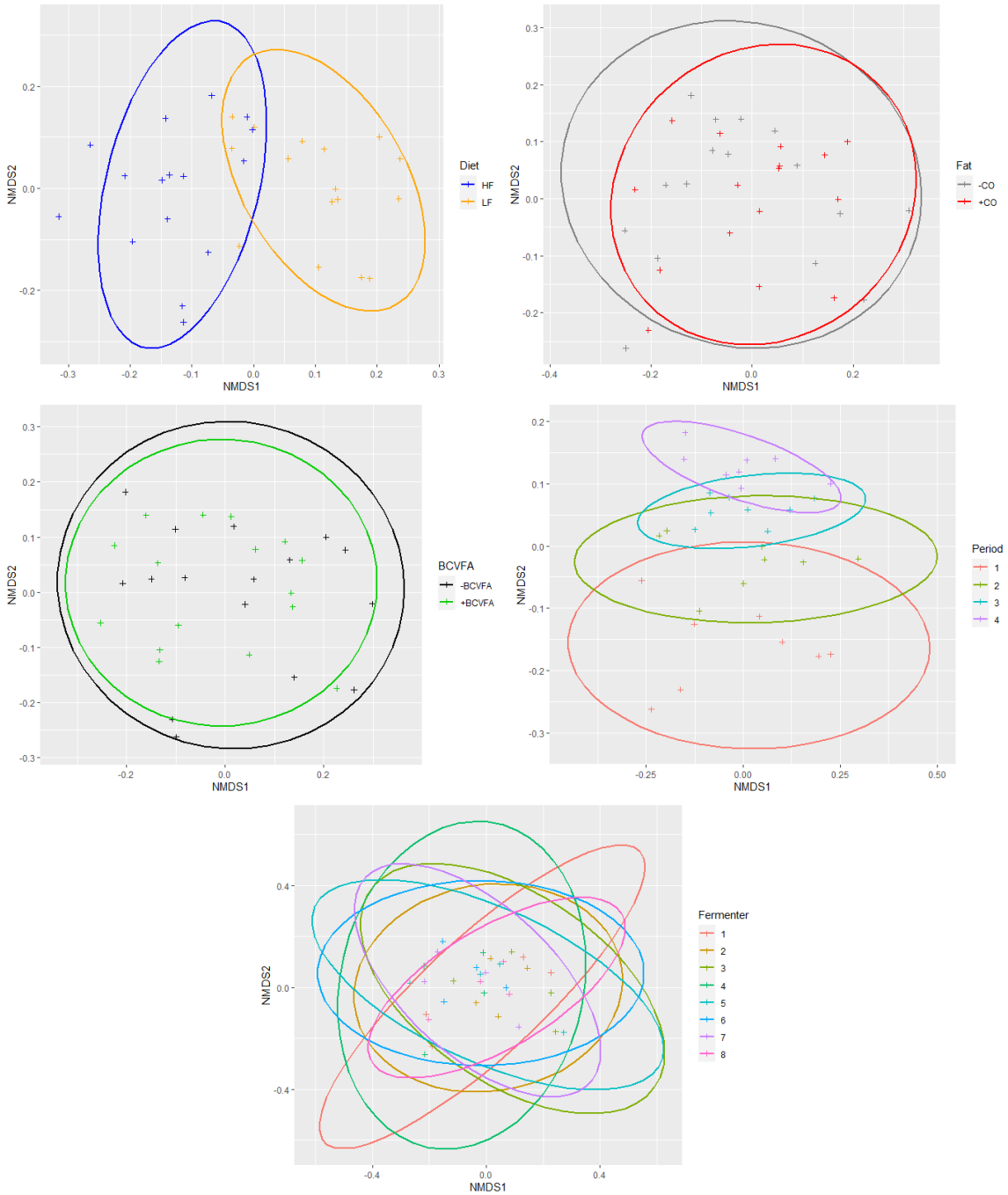

Figure 4.2 Nonmetric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity of 16S rRNA gene relative abundance (rarefied data) from continuous cultures that were fed high (HF) or low forage (LF) diets ($P < 0.01$, top left), supplemented without corn oil (– CO, top right) or with corn oil (+ CO, $P = 0.40$), and without branched-chain VFA (–BCVFA, middle left) or with BCVFA (+ BCVFA, $P = 0.98$). The effect of study period (1-4) was $P < 0.01$ (middle right), but Fermenter (bottom) did not affect ($P = 0.96$) the Bray-Curtis dissimilarity. There were no interactions ($P > 0.97$) among treatments.

Discussion

pH and Reduction/Oxidation Potential

There was still a difference in ORP among HF versus LF despite the successful minimization of pH differences under different diet conditions. Roman-Garcia et al. (2021b) fed the same diets but altered pH of buffers infused into continuous cultures and reported that the decreasing pH increased ORP (became less negative). Both pH and ORP are influenced by proton-coupled reduction/oxidation reactions (Krishtalik, 2003). Oxidation/reduction potential is not commonly measured in vivo and, as with pH, measurement of ORP depends on the location in the rumen and the sampling method. The measurement of ORP is sensitive to air contamination, further complicating its measurement (Huang et al., 2018). The ORP was positively correlated to DMI and the concentrate proportion of the diet but negatively correlated with pH, proportion of acetate, and H_2 production (Baldwin and Emery, 1960; Huang et al., 2018; Kelly et al., 2022). The ORP is reflective of intracellular ORP status (Liu et al., 2013), and NAD^+: NADH (or ORP) has regulatory effects on fermentation pathways such as acetate: propionate and H_2 formation via hydrogenases (Hino and Russell, 1985; Bannink et al., 2006; Rodriguez et al., 2006).

Nutrient Degradation

Dual flow continuous cultures have very high starch degradability because of settling of starch granules below the overflow port, as explained by Roman-Garcia et al. (2021b). Because of increased starch, OM degradability also was higher with LF. Higher inclusion of starch is also positively associated with increased apparent ruminal starch digestibility (White et al., 2016). Degradability of NDF should be higher with LF diets because of increased inclusion of NDF sources such as soybean hulls that are more degradable than forage NDF. We expected that CO, especially with LF diet, would have a bacteriostatic effect on cellulolytics, but the interaction of diet × CO was contrary to what was expected and alleviated a limitation in NDF degradability in the LF – CO diet. The 3% additional CO, designed to provide approximately 1.5% of DM as linoleic FA, was not designed to be excessive but has shown to decrease total tract NDF degradability only moderately (Weld and Armentano, 2017). Though other studies with similar or lower linoleic FA inclusion have induced milk fat depression, those have been with other factors such as low forage, which would decrease pH in vivo (Baldin et al., 2018), especially when combined with decreased particle size, which in addition to decreasing effective NDF would increase passage rate from the rumen compared with coarser forage (Ramirez Ramirez et al., 2016). In our study with controlled pH and passage rate, cellulolytic bacteria presumably had less inhibition with additional CO supplementation compared with potential indirect effects in vivo. Moreover, the benefit from CO supplementation with LF could be due to the inclusion of oleic and palmitic FA included in corn oil, which in vivo has benefited NDF degradability (de Souza et al., 2021). However, with the HF diet, there

was no increase in NDF degradation with CO, likely due to a lower than expected FA increase with the HF diet when CO was added (Table 4.1). Therefore, the increased NDF degradation with CO and LF likely was due to greater provision of oleic and palmitic FA than CO supplementation with HF diet.

Increased NDF degradation is a frequently reported benefit of BCVFA supplementation (Liu et al., 2018b; Wang et al., 2019; Roman-Garcia et al., 2021a). Roman-Garcia et al. (2021b) reported a 5.2 percentage unit increase in NDF degradability in continuous cultures with no interactions of BCVFA supplementation with the other factors: high or low pH and high or low passage rate. Wang et al. (2018) noted an interaction of diet and 2-methylbutyrate in which the moderate-concentrate diet benefited from supplementation more than the high-concentrate diet in respect to effective degradability of NDF from corn silage measured in situ. In our study, we expected an interaction because BCVFA was likely in greater demand when fermenters were administered the HF diet due to the higher relative abundance of populations with known BCVFA requirements or high proportions of branched-chain lipids in their structure: i.e. *Butyrivibrio, F. succinogenes,* and *R. flavefaciens* (Stewart et al., 1997). The LF treatments had a higher abundance of *P. ruminicola*; although many strains do not require BCVFA, *P. ruminicola* subsp. *brevis* was characterized as requiring 2-methylbutyrate (Dehority, 1966). However, with the LF diet, the cellulolytics, even though they are a smaller proportion of the population, likely have more competition for BCVFA with amylolytics that do not require them but can use them (Firkins, 2010). In our study, the lack of interactions between BCVFA and dietary factors for NDF degradation could indicate a

general need for adequate BCVFA under various conditions or could be a result of lack of statistical power to detect interactions.

Bacterial Protein Synthesis

Both bacterial N and C flows had 3-way interactions, but the ratio of bacterial C: N was decreased with LF compared with HF. A decrease in the ratio would be due to either decreased glycogen storage (decreased C) or increased N concentration from a different bacterial community. Although decreased growth rate from N deficiency typically would increase glycogen, glycogen cycling could prevent accumulation of glycogen (Hackmann and Firkins, 2015b). A more likely explanation is a shift in bacterial populations, type of cell wall, and/or a shift in nucleic acid-N:AA-N (Arambel et al., 1982). Notably Proteobacteria, especially an unknown genus in the Gammaproteobacteria class, increased by a factor of 10 with LF compared to HF, primarily replacing Bacteroidetes. The EMPS differences by diet likely are reflective of amylolytic bacteria having higher maintenance coefficients than fibrolytics (Fox et al., 2004).

In this study there were very few shifts in relative sequence abundance with BCVFA supplementation; therefore, the reported increases in bacterial N were due to an overall increase in bacterial population without major shifts in the prokaryotic profile. Increased microbial N is a commonly reported benefit with BCVFA supplementation. Copies of cells/mL (measured with real-time PCR) increased with supplementation of isobutyrate (Wang et al., 2015), 2-methylbutyrate (Zhang et al., 2015), and isovalerate (Liu

et al., 2014). Additionally, 2-methylbutyrate supplementation in Simmental steers increased purine derivatives excreted in urine (correlated with microbial N flow out of the rumen) but to a greater degree with the moderate-concentrate diet compared to a high-concentrate diet (Wang et al., 2018). Changes in forage:concentrate also changed feed AA profile; increased inclusion of corn typically increases availability of Leu. The availability of cytosolic free BCAA was expected to influence the benefits observed with BCVFA supplementation (Firkins et al., 2015); therefore, the effect of protein supply on microbial N or EMPS benefits with BCVFA in vivo requires further investigation.

VFA production, Gas production, and Isotope Recovery

When pH was decreased with the same forage:concentrate, Roman-Garcia et al. (2021b) reported decreased acetate, increased propionate, and decreased acetate: propionate. Besides differences in pH, decreased forage:concentrate ratio also should increase propionate production by amylolytic bacteria, as demonstrated by our prokaryotic community analysis. For example, *P. ruminicola*, *Succinimonas amylolytica*, and *Succinivibrio* increased with LF, all of which produce succinate (Stewart et al., 1997). Succinate serves as the major precursor for decarboxylation to propionate in the rumen. With supplemental CO, the acetate: propionate ratio tended to decrease. Though there were very minor influences of CO on the prokaryotic profile, both *Ruminobacter* and *S. amylolytica* increased with CO. Both form succinate as an end-product. Additionally, *Coprococcus*, which converts lactate to propionate, increased abundance with CO supplementation (Sheridan et al., 2022).

Though LF increased the abundance of succinate producers, there was not an increase in characterized succinolytic bacteria such as *Selenomonas* or *Succiniclasticum*, which are core bacteria associated with the succinate usage niche (Mizrahi et al., 2021). Regardless, increased propionate production should have decreased methane emission, which was not affected. In contrast, the increased H_2 emission for LF versus HF, as supported by elevated H_2(aq) after feeding, suggests a disruption of methanogenesis that might have been available with the higher carbohydrate degradability of LF. Physical interactions between H_2 producers and archaea (Piao et al., 2014) might have been lessened in these first hours after feeding LF because of the increased abundance of amylolytic bacteria (especially Proteobacteria).

With BCVFA supplementation, the lower acetate and propionate production with both HF and LF is likely a result of increased conversion of degraded C into bacterial C. However, CH_4 production numerically increased with HF when BCVFA was supplemented but decreased when supplemented in LF. Roman-Garcia et al. (2021b) explained increased CH_4 production with BCVFA by the increased NDF degradability but also the potential BCVFA requirements by archaea.

Based on the diet formulation program, HF was predicted to have higher RDP than LH (Table 4.1); however, the opposite was observed in this project (Table 4.2). Increased abundance of some proteolytic bacteria, such as *P. ruminicola*, with LF could account for the difference in RDP. The higher concentrations of BCVFA in the vessels could decrease deamination or decarboxylation due to some sort of feedback inhibition or could be regulating gene expression of, as shown for isovalerate in *P. bryantii* (Trautmann et al.,

2020). The influence of BCVFA supplementation on bacterial AA metabolism needs further investigation.

The BCVFA production (mmol/d) from the vessels were used as a covariate in the model to correct for diet differences in diet AA supply (more Leu with LF) and RDP (greater RDP with LF) to prevent type I error due to greater BCVFA precursor availability with LF versus HF. There were no differences for isotope recovery in the total effluent. Further work is being conducted to determine if the numerical decrease in effluent isotope recovery with LF was the result of catabolism of BCVFA (Chapter 5, Chapter 6). However, with HF, bacteria incorporated 45.6% more label into their components than bacteria with LF, suggesting that HF increased the relative abundance of populations with known BCVFA requirements. Roman-Garcia et al. (2021c) noted that many populations with BCVFA requirements decreased with low pH, but there was no difference in isotope recovery in bacteria. The pH was held constant in our study, supporting the forage:concentrate has a greater role over pH (without acidosis) for BCVFA uptake.

Conclusion

Even though net production of BCVFA decreased with decreasing forage, isotope recovery of the dosed BCVFA did not decrease, supporting greater uptake of BCVFA with increasing fiber in the diet. Although we expected that supplemental BCVFA would be more beneficial with greater forage inclusion, additional BCVFA increased NDF degradability and efficiency of microbial protein synthesis regardless of substrate supply.

A decreasing forage:concentrate probably increases the competition between cellulolytics and amylolytics, so providing supplemental BCVFA are projected to improve bacterial protein supply and feed efficiency by lactating dairy cows under a variety of dietary conditions.

Chapter 5. Effects of branched chain volatile fatty acids supplementation on biohydrogenation and incorporation into microbial lipids in dual flow cultures varying forage and corn oil concentration

Introduction

Microbial membrane homeostasis is mediated through genetic and biochemical mechanisms and is necessary for microbial ability to adjust to different conditions (Kaneda, 1991; Zhang and Rock, 2008). Because desaturation is typically an O_2-using process, anaerobic bacteria, including those in the rumen, rely on a methyl branch in an *iso* and particularly *anteiso* configuration of fatty acids (FA) to increase membrane fluidity. The microbial membrane needs its lipid profile altered to manage fluidity in order to regulate passive permeability and to anchor membrane-bound enzymes. *Eubacterium cellulosolvens* 5494 (a cellulolytic member of the Clostridiales order) increased medium chain FA and unsaturated FA, especially 18:1 derived from the medium, to increase fluidity when switched to cellulose compared to glucose, cellobiose, or fructose as energy sources (Moon and Anderson, 2001). Similarly, both *Fibrobacter succinogenes* and *Ruminococcus albus* decreased their membrane hydrophobicity when exposed to cellulose, and thickening of the cell wall facing the adjacent cellulose was noted for *F. succinogenes* (Burnet et al., 2015).

Cellulolytic bacteria primarily use branched-chain fatty acids (BCFA), which require branched-chain VFA (BCVFA) as a primer, to maintain membrane fluidity (Roman-Garcia et al., 2021c). In addition to cellulolytics, butyrivibrios have high inclusion of BCFA in their membrane structure (Kopecny et al., 2003). Both cellulolytics and butyrivibrios are sensitive to polyunsaturated fatty acids (PUFA), especially butyrivibrios that have a high inclusion of *iso* and *anteiso* BCFA (Maia et al., 2010; Hackmann and Firkins, 2015a). The butyrivibrios, which are well known biohydrogenators, have a very thin membrane structure and greater inclusion of the more fluid BCFA should make them more sensitive to fluidization from PUFA imbedding in the membrane (Hackmann and Firkins, 2015a).

The BCVFA isobutyrate, 2-methylbutyrate, and isovalerate are formed by the degradation of branched-chain AA (BCAA) are primers for synthesis of *iso* even-chain, *anteiso* odd-chain, and *iso* odd-chain FA, respectively (Parsons and Rock, 2013). In addition to BCFA synthesis, Allison et al. (1962b) documented that *Ruminococcus flavefaciens* incorporated ^{14}C from isovalerate into a C15 aldehyde (ALD). Additionally, *R. albus* incorporated ^{14}C from isobutyrate into C14 and C16 ALD. The authors presumed that the ALD with ^{14}C label were branched-chain aldehydes (BCALD) from plasmalogens. Plasmalogens are formed by the reductive conversion of an ester bond to a vinyl ether bond in the sn-1 position of a phospholipid in anaerobic bacteria (Jackson et al., 2021).

Unlike alkaline hydrolysis (Allison et al., 1962b), acid hydrolysis can release plasmalogen components. During the standard methylation process to prepare for GC analysis, FA are converted to FA methyl esters (FAME), but the vinyl ethers in

plasmalogens are converted to dimethylacetals (DMA). With GC analysis, individual DMA and FAME can coelute. The ALD are a much small proportion of bacterial lipids and have few commercial standards, which would underrepresent quantification of ALD. These DMA can be important products in mixed ruminal bacteria (Alves et al., 2013) and sink for BCVFA precursors (Allison et al., 1962b), but currently their role is not defined and needs further exploration (Goldfine, 2017). As documented therein, plasmalogen lipids are more tightly packed than phospholipids, which decreases the permeability of membranes. An emerging role for disruption of reactive oxygen damage in anaerobic bacteria has not been verified in rumen bacteria, but prevalence is likely based on a high proportion of genes in plasmalogen synthesis recovered after searching genomes from bacteria in Firmicutes and Actinobacteria compared with Bacteroidetes and Proteobacteria (Jackson et al., 2021). Thus, BCVFA as precursors likely play an emergingly important role in rumen bacterial plasmalogens with branched lipids.

Our objective was to investigate the effect of dietary conditions and BCVFA supplementation on lipid metabolism especially influences on BCL, both BCFA and BCALD, from bacteria harvested from dual flow continuous cultures. Supplemental BCVFA was expected to increase the extent of biohydrogenation of supplemented PUFA because butyrivibrio could benefit from increased supply of BCVFA primers for BCFA synthesis. Additional PUFA in the diet was expected to decrease BCL in bacteria and decrease BCVFA usage for BCL synthesis, because of PUFA inhibition of cellulolytic and butyrivibrio bacteria. Increasing forage in the diet was hypothesized to increase the incorporation of supplemented BCVFA into bacterial BCL because of the greater

proportion of cellulolytic bacteria in the microbial population; cellulolytics likely have more BCL than noncellulolytics (Miyagawa, 1979; Or-Rashid et al., 2007).

Material and Methods

Experimental Design and Treatments

The experiment was a 2 × 2 × 2 factorial arrangement of 8 treatments of high (HF, 67:33) or low (LF, 33:67) forage:concentrate, without or with 3% corn oil (CO), and without or with dosed BCVFA supplementation (2.15 mmol/d each of isobutyrate, isovalerate, and 2-methylbutyrate). Dietary conditions are described in Chapter 4 and FA concentration and profile are presented in Table 5.1. Briefly, there were 8 anaerobic dual-flow continuous culture systems administered these 8 treatments in 4 periods in a randomized incomplete block (n = 4).

Background samples were taken on d 5, these were to measure the natural enrichment of ^{13}C in the dual-flow cultures, and samples were also taken during the collection period, which were d 9 through 12. After background sampling (d 5) was completed, [2-methyl-^{13}C]-2-methylbutyrate, [2,3-$^{13}C_2$, 2-methyl-^{13}C]-isobutyrate, and [2,3,4-$^{13}C_3$, 3-methyl-^{13}C]-isovalerate (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) was dosed and replace a portion of the unlabeled source to provide equivalent to 5 mg/d ^{13}C from each BCVFA. Doses were provided through d 12. The source of labeled 2-methylbutyrate is racemic.

Table 5.1 Fatty acid composition of the diets varying in forage and corn oil supplementation.

Item	HF – CO[1]	HF + CO	LF – CO	LF + CO
FA, % DM[2]	2.69	3.71	2.70	4.01
FA Profile, g/100 g total FA				
12:0	0.0124	0.0558	0.00625	0.0109
14:0	0.955	0.705	0.880	0.535
15:0	0.133	0.107	0.115	0.0773
16:0	34.0	29.7	37.1	29.5
16:1 *cis*-9	0.522	0.379	0.261	0.176
17:0	1.71	4.09	1.97	1.64
18:0	10.9	12.0	10.6	7.44
18:1 t*rans*-9	1.41	1.05	0.711	0.479
18:1 *cis*-9	17.7	17.7	20.5	21.0
18:2 *cis*-9, *cis*-12	11.7	18.0	16.3	30.9
18:3 *cis*-6, *cis*-9, *cis*-12	11.9	8.58	6.42	4.55
20:0	4.18	3.16	2.29	1.59
21:0	0.536	0.494	0.364	0.247
22:0	1.29	1.10	0.707	0.535
24:0	0.484	0.998	0.360	0.450
24:1-*cis* 9	1.35	0.978	0.677	0.456
Other	1.22	0.882	0.611	0.412

[1] Treatments are high forage (HF, 67% forage) or low forage (LF, 33% forage) and – CO (no additional supplemented fat) or + CO (3% DM supplemented fat as corn oil).

[2] Fatty acids (FA) were analyzed by GC and compare with predicted values: 2.97 5.40, 3.28, and 5.91% of DM for HF – CO, HF + CO, LF – CO, and LF + CO, respectively, by Spartan Ration Evaluator (v 3.0.3, Michigan State University, Department of Animal Science).

Sample Collection and Processing

Effluents from the continuous cultures were collected on ice to prevent microbial activity outside of the dual-flow system on d 9 to 12. Every 12 h before feeding, both filtrate and overflow effluents were weighed and mixed. Duplicate 250-mL samples were subsampled One 250-mL subsample was dosed with 8 mL of 5 *N* HCl to bring the pH down to 2 and stored at 4°C for 24 h before differential centrifugation to recover a bacterial sample that was enriched with particulate-associated bacteria (Whitehouse et al., 1994) and

lyophilized. The other 250-mL subsample was stored at -20°C and pooled daily, and lyophilized. The bacteria pellet and lyophilized effluent samples were pooled by fermenter over all days within the collection period for analysis.

Approximately 0.50 g of lyophilized effluent or feed samples and 0.10 to 0.20 g of bacteria pellet were methylated as described by Sukhija and Palmquist (1988) with the adaptations described by Jenkins (2010). In addition, 17:1 cis-10 heptadecenoic acid (Sigma-Aldrich, Laramie, WY) was the internal standard, replacing 17:0 or 19:0 as standards because bacteria also synthesize small amounts of these FA. With every period of bacteria pellet, 2 samples of octadecanal standards (18:0 ALD, Apollo Scientific, Bredbury, United Kingdom) were also methylated to serve as a DMA standard.

Separation of FAME and DMA

The separation of FAME and DMA was only performed on methylated samples from bacteria pellets. Thin-layer chromatography (TLC) was used to separate the FAME and DMA according to standard procedure (Alves et al., 2013). The addition of 75 mL of methylene chloride and a 1 h incubation period allowed for full saturation of the TLC chamber. Nitrogen was used to evaporate 0.5 mL of each sample. Samples were redissolved in 200 μL of methylene chloride and added to individually labeled lanes on glass TLC plates one drop at a time. On each plate lane 1 was 17:1 cis-10-heptadecenoic acid methyl ester (Sigma-Aldrich, Laramie, WY), lanes 2 to 5 were samples, and the final lane was the DMA standard (Figure 5.1). The TLC plate was placed in the saturated chamber for approximately 30 min, allowing the solvent to travel 10 to 15 cm vertically on

the plate. Once removed, the TLC plate was dried with N_2 gas and sprayed with 0.1% 2', 7'-dichlorofluoroscein in methanol (w/v). An ultraviolet lamp at 254 nm was used to illuminate and identify the FAME and DMA spots for collection, lane 2 to 6 on each plate were collected.

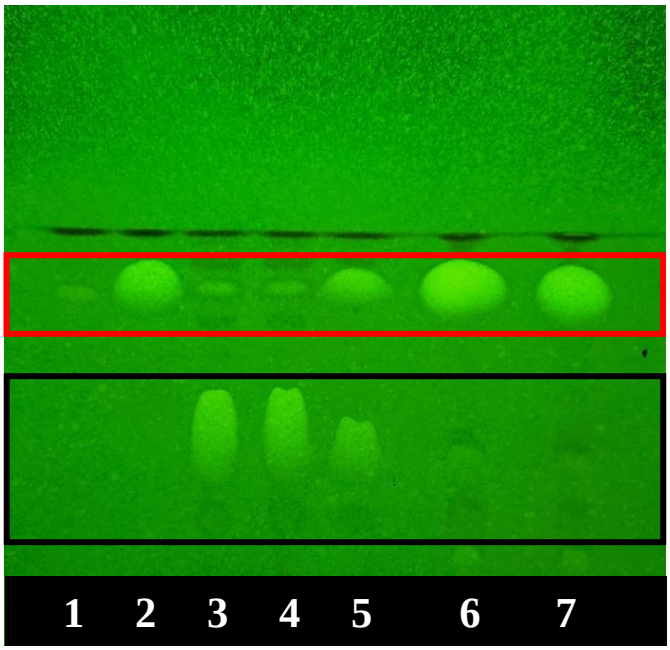

Figure 5.1. Example thin layer chromatography plate used in the separation of fatty acid methyl esters (FAME, red rectangle) and dimethylacetals (DMA, black rectangle). Lanes 1 and 2 are different concentrations of cis-10 heptadecenoic acid (Sigma-Aldrich, Laramie, WY). Lanes 3 and 4 contained octadecanal standards (Apollo Scientific, Bredbury, United Kingdom), which has minor contamination in the FAME band. Lane 5 was a mix of the 17:1 standard and 18:0 aldehyde. Lanes 6 and 7 were from methylated extracts from approximately 0.15 g of lyophilized bacteria pellet. All samples were methylated following the procedure described by Jenkins (2010) and then separated via TLC as described by Alves et al. (2013).

Fatty acid methyl esters and DMA for each sample were outlined in pencil and scraped from the TLC plate into individual collection tubes. Samples had 1.5 mL of methanol added and were sonicated for 10 min. Following the addition of 2 mL of hexane and 1.5 mL of 5% NaCl, samples were vortexed and centrifuged at 400 × g for 5 minutes. The organic phase was removed from each tube and transferred to a new tube. With the addition of another 2 mL of hexane, centrifugation and organic phase collection was repeated. Solvent was removed via evaporation under nitrogen gas. The FAME samples were redissolved with 1 mL of hexane and added to GC vials. The DMA samples were redissolved with 0.2 mL of hexane with 17:1 cis-10-heptadecenoic acid methyl ester, which provided approximately 90 ug of methylated standard. The 17:1 was added back into the DMA samples, including the DMA standard samples from lane 6, to serve as the internal standard to correct for injection variation. The DMA samples were only redissolved with 0.2 mL of hexane to ensure the DMA was concentrated enough to allow for adequate peaks during gas chromatography analysis.

Gas Chromatography and Isotope Ratio Mass Spectrometry Procedure

Effluent and feed FAME samples were injected into a Hewlett-Packard 5890 GC (Agilent Technologies, Santa Clara, CA) equipped with a SP-2560 capillary column (100 m × 0.25 mm × 2.0 μm). The carrier gas (He) flow was 20 cm/s with a 20:1 split the inlet temperature was 280°C and the detector temperature was 280°C. The initial oven temperature was 60°C and held for 4 min, the temperature was then increased 25°C/min to 150°C and held for 20 min. The temperature was then raised to 160°C, 1°C/min, and held

for 50 min, or until 18:1 isomers were eluted. The temperature was increased at 5°C/min to 240°C and was held for 20 min.

Bacterial FAME, DMA, and DMA standards were injected into a GC (Trace 1300; Thermo Fisher Scientific) with a SP-2560 capillary column (100 m × 0.25 mm × 2.0 µm) and equipped with isotope-ratio MS (Delta V Advantage; Thermo Fisher Scientific). Methyl esters were separated using He as a carrier at 20 cm/s. The injector temperature was 280°C with splitless mode; initial oven temperature was 60°C and held for 4 min, the temperature was then increased 50°C/min to 150°C and held for 20 min. The temperature was then raised to 170°C, 1°C/min, and held for 30 min. The final temperature increase was 5°C/min to 240°C and was held for 5 min. The sample was then combusted through a combustion reactor as described by Roman-Garcia et al. (2021a). Isotope-ratio MS was calibrated for ^{13}C enrichment using standards of FAME with known ^{13}C enrichment (n18M, USGS71, and USGS72; Reston Stable Isotope Laboratory).

Individual FAME were identified using the standard mix GLC-68D (Nu-Check Prep Inc., Elysian, MN), GLC-110 (Matreya LLC, State College, PA), and single FAME standards 18:1 t11 and 18:2 t10 c12 (Nu-Check Prep Inc., Elysian, MN). Standard mixes of Bacterial Acid Methyl Esters CP Mixture (Matreya LLC, State College, PA) and 37 Component FAME Mix (Sigma-Aldrich, Laramie, WY) were used for peak identification and FAME response factor calculations (area/concentration). There were 2 samples that were identified as having 16:0 and 18:0 FA in the DMA fraction, the TLC procedure was repeated for these samples.

Chromatograms from Molkentin and Precht (1995) and Alves et al. (2013) were used for the order of elution of 18:1 isomers and DMA, respectively. For DMA, there were no commercially available appropriate internal standards or standard mixes at the time we performed this research to quantify bacterial DMA. Even though 18:0 ALD standard was available this was an inappropriate internal standard because 18:0 ALD is present in bacteria and was influenced by dietary conditions (Alves et al., 2013; Ventto et al., 2017; Mannelli et al., 2018). Therefore, the 18:0 ALD standards, which were methylated separated from the bacteria samples and were used for response factor calculations and to account for DMA loss during methylation and the TLC separation procedure (Figure 5.2). First, the DMA standards were used to determine a response factor for 18:0 DMA (area/concentration). The 18:0 ALD standard was not pure, but the 18:0 and 18:1 FA impurities were quantified, as described previously, and were accounted for in our calculations. Then the ratio of the response factor acquired for 18:0 DMA: 18:0 FAME was used as a conversion factor on the other FAME response factors to convert them to the corresponding DMA response factors. This also accounted for sample loss during methylation. Finally, the DMA standards collected after the TLC plate procedure were used as a recovery factor for the sample loss during TLC plate separation.

In a separate study, BCL were further confirmed as either *iso* even, *iso* odd, or *anteiso* lipids from bacterial pellets from dual flow continuous cultures that were individually dosed ^{13}C labeled isobutyrate, isovalerate, 2-methylbutyrate, Val, Leu, and Ile (data not shown). The single dosage confirmed that BCALD identified as above were indeed derived from (without crossover) the respective parent compounds.

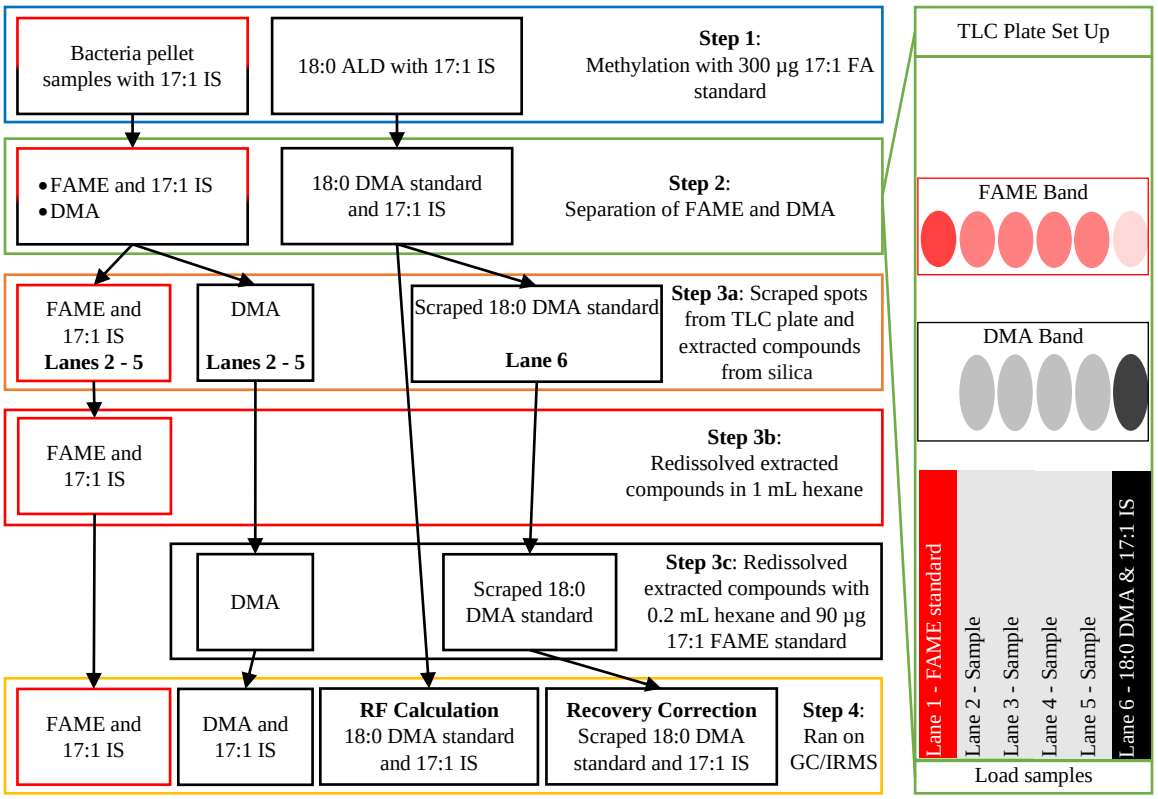

Figure 5.2. Flow chart describing the procedure for methylation of fatty acids (FA) and vinyl ether in plasmalogens and the separation of fatty acid methyl esters (FAME, red boxes) and dimethylacetals (DMA, black solid boxes).
Diagrammed in step 1, bacteria pellets and 18:0 aldehyde (ALD) standard were methylated with 300 µg of cis-10 heptadecenoic acid (17:1) internal standard (IS). In step 2, the FAME, DMA, and 18:0 DMA standard were separated using thin layer chromatography. The FAME standard was only used for verification of the procedure; the spot in lane 1 was not collected. In step 3a, spots in lanes 2 to 6 were identified, scraped, and extracted from the silica. In step 3b, the FAME samples with 17:1 IS from lanes 2 to 5 were redissolved with 1 mL of hexane. In step 3c, the scraped DMA from lanes 2 to 5 and scraped DMA standard from lane 6 were redissolved with 0.2 mL of hexane that included 90 µg cis-10 heptadecenoic methyl ester. The original 17:1 from methylation was recovered exclusively in the FAME band; therefore, this 17:1 FAME IS is necessary to account for injection error in step 3c. In step 4, the FAME and DMA samples were injected into the GC/IRMS. The 18:0 DMA standards and 17:1 IS were used to calculate a response factor (RF, area/ concentration). The 18:0 ALD standard is not pure, but the but the 18:0 FA and 18:1 FA impurities were quantified, and the 18:0 ALD concentration was corrected accordingly. The RF for 18:0 FAME was calculated using standard mixes of Bacterial Acid Methyl Esters CP Mixture and 37 Component FAME Mix. The RF for 18:0 ALD was scaled based on the same assumptions for all DMA (e.g., 18:0 FAME RF/18:0 DMA RF × 16:0 FAME RF = 16:0 DMA RF). The scraped 18:0 DMA standard (corrected for impurities) containing the 17:1 IS in step 3c was used to calculate a recovery factor for DMA from each TLC plate ran [sample DMA concentration/(actual concentration/expected concentration)].

The methylation procedure adds a methyl group per FA and 2 methyl groups per ALD to form the respective FAME and DMA (Figure 5.3). Therefore, FAME and ALD flows were converted to FA (FAME mg/d × FAME M.W. / FA M.W.) and ALD flow (DMA mg/d × DMA M.W. / ALD M.W.), where M.W. is the molecular weight. Biohydrogenation index was calculated as described by Tice et al. (1994) $100 - [100 \times ((18{:}1_{Flow} + (18{:}2_{Flow} \times 2) + (18{:}3_{Flow} \times 3))/18_{Flow})/((18{:}1_{Intake} + (18{:}2_{Intake} \times 2) + (18{:}3_{Intake} \times 3))/18_{Intake}]$, where 18:1 is all C18 FA with 1 unsaturated bond, 18:2 is all C18 FA with 2 unsaturated bonds, 18:3 is all C18 FA with 3 unsaturated bonds, and 18 is the total C18 FA with or without unsaturated bonds. Respective bacterial N flows were converted to FA and ALD flows based on the FA:N of ALD:N ratios of bacterial samples. Recovery of ^{13}C in bacterial lipid was calculated as the product of atom percent excess (APE, ^{13}C atom percent - background ^{13}C atom percent) and C flow of FAME or DMA then divided by total ^{13}C dosed and divided by 100. The distribution of recovered dose was the ^{13}C recovered in FA and ALD/total ^{13}C recovered in the bacteria C outflows reported in Chapter 4.

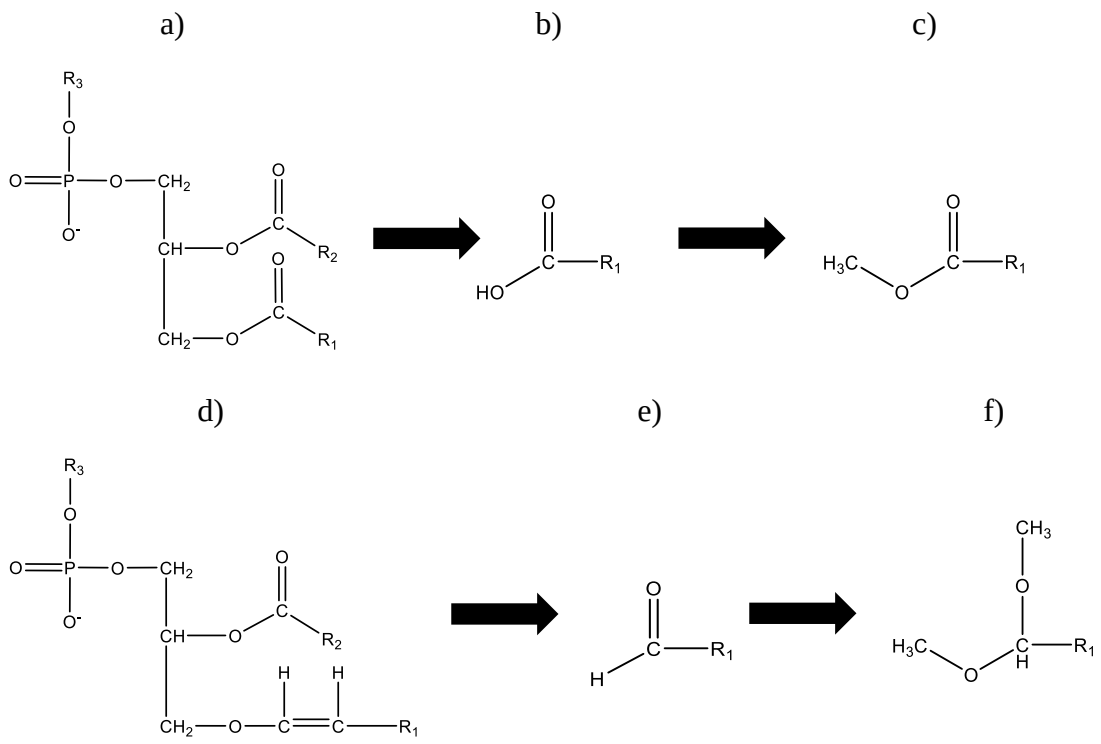

Figure 5.3 Structure of phospholipid (a) and plasmalogen (d) during methylation procedure to free fatty acid (b) and aldehyde (e) before methylation to fatty acid methyl ester (FAME, c) and dimethylacetals (DMA, f).
R_1 and R_2 represent acyl chain lipids (can be a branched-chain or straight chain lipid), while R_3 represents the head group of phospholipid structure (e.g. choline, serine, inositol).

Statistical Analysis

Daily FA flow, bacterial FA and ALD flow, and profile measurements were analyzed using PROC MIXED in SAS 9.4 (SAS Institute Inc.) according to the model described in Chapter 4. Briefly a mixed model had random effects of period and fermenter and fixed effects of diet, CO, BCVFA, and their interactions. Recovery of ^{13}C dose was analyzed with the same model but an additional covariate was included due to LF having a higher RDP than the HF diet and different BCAA supply (Chapter 4). Which would

influence BCVFA pool sizes, therefore BCVFA production (mmol/d) was included as the covariate to account for differing pool sizes.

Results

Biohydrogenation Intermediates and Extent

The FA concentration on a DM basis was greater ($P < 0.01$, Table 5.2) with LF vs HF treatments and with CO supplementation vs without CO. The flows of FA were greater ($P \leq 0.03$) by 14.9% with LF versus HF, 22.4% with CO versus without CO, and by 13.5% with BCVFA supplementation versus without BCVFA. The flows of 18:3 *cis*-6, *cis*-9, *cis*-12 and its biohydrogenation intermediates 18:2 *trans*-11, *cis*-15 and 18:2 *cis*-9, *trans*-11 were greater ($P < 0.01$) with HF diets than LF. However, supplemental CO increased 18:2 *cis*-9, *trans*-11 flow when supplemented with HF but not with LF ($P = 0.04$, Diet × CO). The flows of 18:2 *cis*-9, *cis*-12, total 18:1 isomers, 18:1 *trans*-11, 18:1 *trans*-10, and 18:0 all were greater ($P < 0.01$) with LF versus HF. The flow of 18:1 *trans*-10 increased with BCVFA supplementation, but the increase tended to be more with LF than HF treatments ($P = 0.10$, Diet × BCVFA). Supplemental CO tended to increase ($P = 0.08$) the flows of linolenic and increased ($P < 0.01$ each) the flow of 18:2 *trans*-10, *cis*-12, total 18:1 isomers, 18:1 *trans*-11, 18:1 *trans*-10, and stearic FA. Supplementing BCVFA tended to increase ($P = 0.06$) the flows of linoleic and increased ($P \leq 0.05$) the flow of 18:2 *trans*-10, *cis*-12, total 18:1 isomers, 18:1 *trans*-11, and stearic FA.

Extents of biohydrogenation of linolenic and linoleic acids increased ($P \leq 0.02$) with CO supplementation and tended to decrease ($P = 0.07$ each) with BCVFA supplementation (Table 5.2). However, CO interacted ($P = 0.03$) and BCVFA tended ($P = 0.10$) to interact with diet. Biohydrogenation of linoleic acid increased more when CO was supplemented with LF diets. Biohydrogenation of linoleic acid decreased when BCVFA were supplemented to LF diets but did not influence biohydrogenation with HF treatments. Biohydrogenation index, which was calculated as a percentage of unsaturated bonds that were saturated in the vessels, increased ($P < 0.01$) with LF versus HF and with CO versus without supplemental CO.

Table 5.2 Effluent fatty acid flows and biohydrogenation of PUFA in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation.

	HF[1]				LF				SEM	Significance[2]		
	– CO		+ CO		– CO		+ CO					
	–	+	–	+	–	+	–	+		Diet	CO	BCVFA
Item	BCVFA	BCVFA	BCVFA	BCVFA	BCVFA	BCVFA	BCVFA	BCVFA				
FA flow, mg/d												
18:3 *cis*-6, *cis*-9, *cis*-12	103	98.5	90.7	89.5	53.4	67.7	51.0	53.3	11.8	< 0.01	0.13	0.64
18:2 *cis*-9, *cis*-12[3]	110	92.4	124	129	167	194	184	224	21	< 0.01	0.04	0.24
18:2 *trans*-11, *cis*-15	4.67	3.71	4.84	5.74	1.50	2.74	2.43	2.63	1.12	< 0.01	0.19	0.54
18:2 *cis*-9, *trans*-11[4]	18.3	20.7	27.1	25.2	18.0	14.9	15.5	15.3	3.4	< 0.01	0.12	0.68
18:2 *trans*-10, *cis*-12	1.00	1.00	3.58	4.30	1.36	2.75	4.11	5.09	0.93	0.05	0.08	0.07
18:1 *trans*-11	98.0	99.4	225	224	190	192	301	374	25.5	< 0.01	< 0.01	0.15
18:1 *trans*-10[3]	11.8	12.4	28.1	28.6	30.1	33.4	43.2	55.5	3.3	< 0.01	< 0.01	0.05
18:0	582	597	1000	952	1032	1091	1267	1552	113	< 0.01	< 0.01	0.21
BH[5]	44.6	48.0	52.9	51.3	53.5	51.9	59.7	60.7	2.4	< 0.01	< 0.01	0.83

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] *P* values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] Interaction of Diet × BCVFA supplementation has $P \leq 0.10$.

[4] Interaction of Diet × CO supplementation has $P = 0.03$.

[5] Biohydrogenation (BH) = $100 - [100 \times ((18{:}1_{Flow} + (18{:}2_{Flow} \times 2) + (18{:}3_{Flow} \times 3))/18_{Flow})/((18{:}1_{Intake} + (18{:}2_{Intake} \times 2) + (18{:}3_{Intake} \times 3))/18_{Intake}]$, 18 = total C18 FA (Tice et al., 1994).

Bacterial Fatty Acid Concentration and Profile

The FA concentration of bacteria were on an OM basis because of ash contamination during bacterial harvesting (Table 5.3). Fatty acid concentration tended to be lower ($P = 0.10$) with HF at 9.25% compared to LF at 10.7%, but bacterial FA flow was not influenced by any main effect or interactions. The weight percentage of even straight-chain FA (ECFA) decreased when CO was supplemented but to a greater degree in HF treatments ($P = 0.01$, Diet × CO). Supplemental BCVFA also decreased ($P < 0.01$) ECFA profile. Both 12:0 and 14:0 profile was lower ($P \leq 0.01$) with LF versus HF. The weight percentage of 14:0 and 16:0 decreased ($P \leq 0.06$) with BCVFA supplementation. Profile of 18:0 decreased ($P = 0.05$) with CO. Arachidonic acid (20:0) decreased with CO supplementation but more with HF ($P = 0.04$, Diet × CO). The profile of odd straight-chain FA (OCFA) was lower ($P < 0.01$) with LF versus HF and decreased ($P = 0.01$) with CO versus without CO. Although there were no interactions with OCFA, 15:0 FA, the major OCFA, decreased when CO was supplemented but more with LF than HF diets ($P = 0.07$, Diet × CO). Additionally, 17:0 concentration was lower ($P < 0.01$ each) with LF versus HF, with CO versus without CO and tended to decrease ($P = 0.09$) with BCVFA versus without BCVFA. Also, 19:0 FA was lower ($P < 0.01$) with LF versus HF and decreased ($P = 0.06$) with BCVFA versus no BCVFA. The total 18:1 isomers in the bacterial FA profile increased ($P < 0.01$ each) from 14.7% of total FA with HF to 18.5% with LF diets, from 14.7% without CO to 18.5% with CO, and from 15.4% without BCVFA to 17.8% with BCVFA. The major 18:1 isomers (18:1 *trans*-11 and 18:1 *cis*-9) also were greater ($P \leq 0.05$) with LF versus HF, with CO versus no CO, and with BCVFA versus no BCVFA.

The 18:1 *trans*-10 weight percentage did not experience any main effects but increased when BCVFA were supplemented in HF diets but decreased when BCVFA were supplemented with LF ($P = 0.09$, Diet × BCVFA). The concentration of linoleic acid in bacterial FA was tended to be greater ($P = 0.09$) with LF versus HF, increased ($P = 0.03$) with BCVFA versus without BCVFA, but was unaffected by CO.

The total BCFA was greater ($P < 0.01$) with HF, 9.46% of total FA, compared to LF, 7.06% (Table 5.3). The total *iso* even-chain BCFA also was greater ($P < 0.01$) with HF at 1.72% versus LF at 1.09% and tended to decrease ($P = 0.10$) from 1.53% without CO to 1.28% with CO. The percentages of FA as 12:0 *iso*, 14:0 *iso*, and 16:0 *iso* were lower ($P < 0.01$) with LF compared to HF. Both 14:0 *iso* and 18:0 *iso* decreased ($P \leq 0.04$) with CO supplementation. When BCVFA were supplemented, 12:0 *iso* increased ($P = 0.01$) and 18:0 *iso* tended to increase ($P = 0.06$) in the FA profile. The total *anteiso* BCFA concentration tended to be lower ($P = 0.06$) with HF, 4.64%, compared to LF, 3.95%. The profiles of 13:0 *anteiso* and 17:0 *anteiso* were lower ($P \leq 0.03$) with LF versus HF. The concentration of *iso* odd BCFA was lower ($P < 0.01$) with HF, 3.10%, compared to LF, 2.01%. The percentages of total FA as 15:0 *iso* and 17:0 *iso* were lower ($P \leq 0.01$) with LF versus HF. Supplemental BCVFA tended to decrease ($P < 0.10$) 11:0 *iso* and decreased ($P = 0.03$) 17:0 *iso* concentration in bacterial FA.

Table 5.3. Bacterial fatty acid (FA) content, flow rate, and FA profile in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation.

	HF[1]				LF				SEM	Significance[2]		
	– CO		+ CO		– CO		+ CO					
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA
FA, % OM	9.85	9.14	9.62	8.39	11.2	9.05	10.7	11.9	1.35	0.10	0.68	0.40
Total FA flow, mg/d	1035	1168	1066	930	1136	983	1122	1237	124	0.41	0.92	0.90
Profile, g/100 g of total FA												
∑ ECFA[3, 4]	70.0	67.6	63.9	63.3	68.7	64.9	67.6	63.8	1.3	0.94	< 0.01	< 0.01
12:0	0.402	0.416	0.514	0.477	0.572	0.651	0.655	0.612	0.103	0.01	0.34	0.96
14:0	3.34	2.92	3.90	3.05	4.25	4.03	4.43	3.91	0.39	< 0.01	0.46	0.06
16:0	27.7	22.0	24.5	23.7	26.6	25.3	26.5	24.5	1.7	0.21	0.54	0.02
18:0	38.1	41.6	34.6	35.7	36.9	34.6	35.7	34.6	2.4	0.13	0.05	0.83
20:0[3]	0.517	0.670	0.386	0.367	0.309	0.339	0.316	0.219	0.062	< 0.01	< 0.01	0.65
∑ OCFA[5]	5.64	5.18	5.31	5.05	4.40	4.41	3.63	3.41	0.57	< 0.01	0.01	0.27
11:0	0.0248	0.0240	0.0250	0.0401	0.0183	0.0244	0.0258	0.0271	0.0081	0.39	0.22	0.31
13:0	0.222	0.196	0.250	0.225	0.209	0.194	0.199	0.175	0.028	0.11	0.69	0.21
15:0[3]	3.73	3.21	3.48	3.21	2.90	2.83	2.27	2.03	0.46	< 0.01	0.01	0.09
17:0	0.929	0.832	0.746	0.753	0.750	0.659	0.616	0.582	0.068	< 0.01	< 0.01	0.09
19:0	0.763	0.939	0.834	0.858	0.536	0.730	0.539	0.626	0.087	< 0.01	0.65	0.06
∑ 16:1	0.425	0.458	0.515	0.509	0.462	0.419	0.453	0.410	0.077	0.63	0.39	0.99
16:1 *cis*-7	0.144	0.141	0.181	0.130	0.093	0.089	0.084	0.153	0.045	0.15	0.50	0.92
16:1 *cis*-9	0.230	0.207	0.294	0.303	0.298	0.212	0.299	0.272	0.054	0.74	0.12	0.36
16:1 *trans*-3	0.0507	0.1108	0.0397	0.0752	0.0711	0.1169	0.0698	0.0454	0.0348	0.76	0.18	0.19
∑ 18:1	11.2	13.6	16.0	17.9	16.0	18.1	18.4	21.6	1.3	< 0.01	< 0.01	< 0.01
18:1 *trans*-4[3, 6]	0.0341	0.0604	0.0878	0.0824	0.0836	0.0741	0.0913	0.0972	0.0099	< 0.01	< 0.01	0.49
18:1 *trans*-5[7]	0.0244	0.0447	0.0492	0.0500	0.0454	0.0535	0.0653	0.0637	0.0055	< 0.01	< 0.01	0.04
18:1 *trans*-6 and *trans*-8	0.365	0.477	0.592	0.665	0.576	0.706	0.750	0.887	0.073	< 0.01	< 0.01	0.02

continued

Table 5.3 continued

Item	HF[1] – CO – BCVFA	HF[1] – CO + BCVFA	HF[1] + CO – BCVFA	HF[1] + CO + BCVFA	LF – CO – BCVFA	LF – CO + BCVFA	LF + CO – BCVFA	LF + CO + BCVFA	SEM	Significance[2] Diet	CO	BCVFA
18:1 *trans*-9	0.234	0.273	0.417	0.458	0.308	0.416	0.394	0.560	0.059	0.07	< 0.01	0.03
18:1 *trans*-10[8]	0.410	0.530	0.587	0.783	0.634	0.617	0.783	0.483	0.142	0.57	0.23	> 0.99
18:1 *trans*-11	3.36	4.15	5.02	5.47	4.15	4.82	5.03	6.26	0.56	0.05	< 0.01	0.01
18:1 *trans*-12	0.420	0.516	0.735	0.806	0.558	0.663	0.757	0.918	0.053	< 0.01	< 0.01	< 0.01
18:1 *cis*-9	3.67	4.49	4.79	5.49	4.30	5.15	4.70	6.04	0.33	0.05	< 0.01	< 0.01
18:1 *trans*-15 and *cis*-11	1.95	2.01	2.49	2.61	4.38	4.39	4.59	4.74	0.50	< 0.01	0.13	0.76
18:1 *cis*-12[3, 8]	0.363	0.428	0.693	0.772	0.495	0.636	0.614	0.889	0.048	< 0.01	< 0.01	< 0.01
18:1 *cis*-13[8]	0.0385	0.0294	0.0517	0.0630	0.0438	0.0532	0.0480	0.0758	0.0066	0.04	< 0.01	0.04
18:1 *trans*-16 and *cis*-14	0.262	0.410	0.406	0.465	0.341	0.391	0.431	0.466	0.044	0.41	< 0.01	0.01
18:1 *cis*-15	0.0489	0.0723	0.0743	0.0775	0.0553	0.0614	0.0722	0.0708	0.0099	0.55	0.02	0.17
18:1 *cis*-16	0.0176	0.105	0.0383	0.0824	0.0330	0.0526	0.0475	0.0514	0.0205	0.19	0.80	< 0.01
18:2 *cis*-9, *cis*-12	1.27	1.54	1.42	1.71	1.60	1.98	1.51	1.75	0.20	0.09	0.98	0.03
∑ BCFA[9]	9.21	8.99	10.43	9.20	7.01	7.89	6.39	6.94	1.12	< 0.01	0.95	> 0.99
∑ *iso* even-chain	1.65	1.92	1.73	1.60	1.06	1.51	0.873	0.931	0.268	< 0.01	0.10	0.29
12:0 *iso*	0.0105	0.0157	0.0114	0.0143	0.00330	0.00991	0.00484	0.00604	0.00342	< 0.01	0.59	0.01
14:0 *iso*	0.863	0.963	0.861	0.641	0.603	0.824	0.488	0.516	0.150	0.02	0.04	0.71
16:0 *iso*	0.699	0.837	0.809	0.856	0.375	0.581	0.329	0.358	0.124	< 0.01	0.60	0.13
18:0 *iso*	0.0817	0.101	0.0506	0.0865	0.0810	0.0925	0.0509	0.0506	0.0136	0.20	< 0.01	0.06
∑ *anteiso* odd-chain	4.78	4.23	5.11	4.43	4.20	4.10	3.68	3.83	0.57	0.06	0.85	0.40
11:0 *anteiso*	0.00237	0.00276	0.00236	0.00534	0.00264	0.00571	0.00353	0.00383	0.00357	0.54	0.73	0.16
13:0 *anteiso*	0.0282	0.0322	0.0404	0.0445	0.0218	0.0327	0.0276	0.0286	0.0057	0.03	0.09	0.19
15:0 *anteiso*	4.09	3.51	4.40	3.74	3.69	3.48	3.25	3.30	0.49	0.11	0.95	0.25
17:0 *anteiso*	0.663	0.690	0.664	0.645	0.484	0.582	0.395	0.498	0.086	< 0.01	0.29	0.30

continued

Table 5.3 continued

	HF[1]				LF				SEM	Significance[2]		
	– CO		+ CO		– CO		+ CO					
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA
∑ *iso* odd-chain	2.78	2.84	3.59	3.17	1.75	2.28	1.84	2.17	0.39	< 0.01	0.30	0.62
11:0 *iso*	0.00470	0.00638	0.00474	0.00499	0.00299	0.00839	0.00413	0.00713	0.00303	0.76	0.81	0.10
13:0 *iso*	0.199	0.240	0.266	0.234	0.188	0.258	0.222	0.271	0.029	> 0.99	0.19	0.12
15:0 *iso*	2.11	2.07	2.88	2.45	1.28	1.64	1.39	1.58	0.34	< 0.01	0.21	0.92
17:0 *iso*	0.466	0.531	0.438	0.482	0.277	0.375	0.219	0.311	0.049	< 0.01	0.12	0.03
Other	3.40	4.08	3.73	3.89	3.34	4.18	3.40	3.69	0.33	0.45	0.66	< 0.01

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] *P* values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] Interaction of Diet × CO supplementation has $P \leq 0.07$.

[4] Total even straight-chain fatty acids.

[5] Total odd straight-chain fatty acids.

[6] Interaction of Diet × CO × BCVFA supplementation has $P = 0.07$.

[7] Interaction of CO × BCVFA supplementation has $P = 0.09$.

[8] Interaction of Diet × BCVFA supplementation has $P \leq 0.09$.

[9] Total branched-chain fatty acids.

Bacterial Aldehyde Concentration and Profile

The concentration of ALD in bacteria pellets and total ALD flow had interactions between diet and CO ($P \leq 0.09$, Table 5.4). When CO was supplemented with HF, ALD concentration and flow decreased by 20.1% and 23.6%, respectively, compared to HF – CO treatments, whereas when CO was supplemented with LF, bacterial ALD concentration and flow increased by 15.8% and 13.6, respectively, compared to LF – CO treatments. Overall, the sum of ALD that was straight even-chain ALD (ECALD) was greater ($P < 0.01$, main effect) with LF compared to HF treatments and increased with supplemental BCVFA in HF – CO, HF + CO, and LF + CO diets but not with the LF – CO ($P = 0.08$, Diet × CO × BCVFA). The profile of 14:0 ALD increased with BCVFA supplementation in HF – CO and LF + CO diets but decreased when supplemented with HF + CO and LF – CO ($P = 0.02$, Diet × CO × BCVFA). Supplementation of BCVFA decreased 12:0 ALD but increased both 16:0 and 18:0 ALD in the profile with HF diets, whereas BCVFA with LF did not influence these percentages ($P \leq 0.10$, Diet × BCVFA). The percentage of 16:0 ALD increased with CO supplementation ($P = 0.02$). The percentage that was straight odd-chain ALD (OCALD) tended to increase ($P = 0.07$) with LF compared to HF. Supplementation of BCVFA with HF increased 13:0 ALD percentage but decreased 15:0, whereas with LF treatments 13:0 did not change and 15:0 ALD decreased with BCVFA supplementation ($P \leq 0.02$, Diet × BCVFA). The percentage of 15:0 ALD decreased ($P = 0.03$) with CO supplementation. Finally, 17:0 ALD increased ($P \leq 0.05$) with LF versus HF and with CO versus without CO.

The percentage of total BCALD in the bacterial ALD profile decreased ($P = 0.01$) from 55.4% with HF to 51.4% with LF diets. The total *iso* even BCALD in the profile decreased ($P \leq 0.03$) by 12.2% with LF versus HF and by 8.03% with CO versus without CO. Supplementation of BCVFA increased *iso* 12:0 ALD in the profile with HF – CO and LF – CO but did not influence 12:0 *iso* ALD with HF + CO or LF + CO diets ($P = 0.05$, Diet × CO × BCVFA). The percentage of 16:0 *iso* ALD increased when BCVFA were supplemented to LF diets more than when supplemented to HF diets ($P = 0.07$, Diet × BCVFA). The main effect of CO decreased ($P = 0.04$) 16:0 *iso* with CO versus without CO. The sum of unknown *iso* even BCALD, which were ALD with enrichment that were confirmed with individually dosed ^{13}C labeled Val and isobutyrate (data not shown), was lower ($P < 0.01$) with LF versus HF. The sum of *anteiso* BCALD in bacterial ALD profile was not influenced by any main effects or interactions. The percentages of 11:0 *anteiso* and 13:0 *anteiso* decreased when BCVFA were supplemented with HF treatments, whereas 15:0 *anteiso* and 17:0 *anteiso* increased ($P < 0.10$, Diet × BCVFA). However, BCVFA supplementation with LF 11:0 *anteiso* and 17:0 *anteiso* increased but 13:0 *anteiso* and 15:0 *anteiso* ALD percentages decreased. The sum of unknown *anteiso* BCALD, which were ALD with enrichment that were confirmed with individually dosed ^{13}C-labeled Ile and 2-methylbutyarte (data not shown) were not influenced by any main effects or interactions. The total *iso* odd BCALD in the profile was 11.1% with HF but was 8.95% with LF ($P < 0.01$). The proportion of 11:0 *iso* and 13:0 *iso* decreased with BCVFA supplementation and HF diets but increased with LF diets ($P \leq 0.08$, Diet × BCVFA). When CO was supplemented with HF diets the 11:0 *iso* decreased, but not when CO was added to LF diets ($P = 0.09$, Diet × CO). The percentage of 15:0 *iso* was lower ($P = 0.01$) with LF

versus HF. The profile of 17:0 *iso* ALD increased with BCVFA supplementation in HF – CO, HF + CO and LF – CO treatments but decreased when supplemented with LF + CO and (P = 0.04, Diet × CO × BCVFA). The sum of unknown *iso* odd BCALD, which were ALD with enrichment that were confirmed with individually dosed ^{13}C labeled Leu and isovalerate (data not shown), decreased when BCVFA were supplemented with HF diets, but increased with LF diets (P = 0.07, Diet × BCVFA).

Table 5.4. Aldehyde (ALD) composition from bacteria harvested from continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation.

	HF[1]				LF				SEM	Significance[2]			
	– CO		+ CO		– CO		+ CO						
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA	Diet × BCVFA
ALD, % OM[3]	0.678	0.446	0.391	0.506	0.635	0.533	0.686	0.666	0.107	0.04	0.85	0.31	0.26
ALD flow, mg/d[3]	70.7	56.5	42.9	54.3	65.5	57.9	71.6	68.7	10.5	0.12	0.59	0.59	0.40
Profile, g/100 g of total ALD													
∑ ECALD[4, 5]	19.4	23.3	21.7	23.0	24.4	23.5	25.6	26.1	0.90	< 0.01	0.01	0.03	0.02
12:0	2.25	1.70	2.15	1.64	1.95	1.96	1.84	1.79	0.15	0.66	0.31	0.02	0.03
14:0[5]	5.06	6.28	5.51	5.36	6.02	5.55	5.70	5.90	0.47	0.22	0.58	0.30	0.10
16:0	11.6	15.1	14.0	15.3	16.1	15.6	17.6	17.9	0.9	< 0.01	0.02	0.07	0.06
18:0	0.537	0.893	0.522	0.877	0.543	0.460	0.458	0.571	0.147	0.06	0.99	0.08	0.10
∑ OCALD[6]	6.20	6.26	6.20	7.08	6.83	6.94	7.25	7.36	0.51	0.07	0.24	0.40	0.60
11:0	0.222	0.172	0.194	0.208	0.057	0.185	0.187	0.188	0.060	0.31	0.41	0.58	0.34
13:0	1.61	1.05	1.36	1.09	1.23	1.28	1.18	1.13	0.12	0.33	0.20	0.01	0.02
15:0	2.57	3.42	2.73	3.05	2.46	2.38	2.21	2.02	0.24	< 0.01	0.03	0.02	< 0.01
17:0	1.80	1.62	1.91	2.73	3.09	3.10	3.77	4.02	0.59	< 0.01	0.05	0.50	0.77
∑ 16:1	1.96	1.76	2.02	1.92	1.60	2.10	1.60	1.69	0.26	0.31	0.77	0.66	0.19
∑ 18:1	8.35	7.76	8.14	6.32	8.79	7.46	8.89	8.54	1.65	0.37	0.89	0.24	0.83
∑ BCALD[7]	56.3	55.4	54.8	55.1	52.1	53.4	50.1	50.0	2.1	0.01	0.15	0.91	0.72
∑ *iso* even-chain	24.1	23.1	22.3	22.0	20.2	22.2	18.9	19.0	1.4	< 0.01	0.03	0.76	0.27
12:0 *iso*[5]	4.10	2.60	2.44	2.45	2.05	2.56	1.87	1.89	0.40	< 0.01	0.01	0.32	0.05
14:0 *iso*	14.3	14.3	14.3	13.9	14.1	15.3	13.6	13.8	0.8	0.95	0.20	0.59	0.33
16:0 *iso*	1.96	2.74	1.92	2.33	1.16	1.51	1.08	1.22	0.19	< 0.01	0.04	< 0.01	0.07
18:0 *iso*	0.417	0.312	0.358	0.301	0.336	0.307	0.280	0.155	0.067	0.12	0.16	0.11	0.97
∑ Unknown *iso* even-chain[8]	3.30	3.10	3.23	3.03	2.60	2.62	2.06	2.05	0.40	< 0.01	0.14	0.63	0.60

continued

Table 5.4 continued

	HF[1]				LF				SEM	Significance[2]			
	– CO		+ CO		– CO		+ CO						
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA	Diet × BCVFA
∑ *anteiso* odd-chain	21.1	21.6	20.9	22.1	23.3	22.1	22.5	21.6	1.1	0.20	0.74	0.89	0.20
11:0 *anteiso*	0.100	0.058	0.153	0.074	0.056	0.064	0.070	0.076	0.081	0.06	0.12	0.09	0.04
13:0 *anteiso*	5.61	4.12	5.29	4.69	5.49	5.17	5.14	4.72	0.31	0.30	0.48	< 0.01	0.10
15:0 *anteiso*	11.1	13.6	11.9	13.1	14.6	12.7	13.3	13.1	1.1	0.16	0.77	0.53	0.04
17:0 *anteiso*	0.272	0.462	0.228	0.406	0.139	0.266	0.272	0.192	0.075	< 0.01	0.79	0.01	0.04
∑ Unknown *anteiso* odd-chain	4.00	3.34	3.43	3.81	3.01	3.85	3.74	3.56	0.51	0.75	0.80	0.77	0.47
∑ *iso* odd-chain	11.1	10.7	11.5	11.0	8.71	9.14	8.66	9.28	0.68	< 0.01	0.68	0.98	0.27
11:0 *iso*[3]	0.171	0.141	0.113	0.116	0.0854	0.136	0.0992	0.134	0.0229	0.11	0.24	0.34	0.07
13:0 *iso*	2.87	2.27	2.74	2.45	2.12	2.22	2.21	2.30	0.22	0.02	0.71	0.25	0.08
15:0 *iso*	4.08	4.69	4.60	4.82	3.50	3.55	3.49	4.11	0.45	0.01	0.29	0.19	0.89
17:0 *iso*[5]	0.376	0.365	0.268	0.390	0.394	0.603	0.479	0.292	0.091	0.14	0.21	0.58	0.71
∑ Unknown *iso* odd-chain	3.63	3.23	3.83	3.18	2.62	2.63	2.38	2.45	0.23	< 0.01	0.65	0.12	0.07
Other	7.81	5.53	7.21	6.61	6.26	6.57	6.60	6.36	0.70	0.46	0.74	0.14	0.12

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] *P* values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] Interaction of Diet × CO supplementation has $0.07 \leq P \leq 0.10$.

[4] Even straight-chain aldehydes (ECALD).

[5] Diet × CO × BCVFA interaction has $P \leq 0.08$.

[6] Odd straight-chain aldehydes (OCALD).

[7] Branched-chain fatty aldehydes (BCALD)

[8] BCALD that were unknown but enriched in ^{13}C and confirmed as either *iso* even, *iso* odd, or *anteiso* BCALD with individually dosed ^{13}C labeled sources (isobutyrate, isovalerate, 2-methylbutyrate, Val, Leu, and Ile; unpublished data) in dual flow continuous cultures.

Isotope Recovery in Bacterial Lipids

The recovery of ^{13}C in bacterial lipid C flow was greater ($P < 0.01$, Table 5.5) by 42.2% with HF versus LF diets and CO supplementation decreased recovery with HF but increased recovery with LF ($P = 0.02$, Diet × CO).). There was no appreciable enrichment outside of BCL. Recovery in total BCFA and *anteiso* odd-chain was higher ($P = 0.01$ each) with HF compared to LF, by 48.3% and 42.0% respectively. Dose recovery in *iso* even-chain and *iso* odd-chain only tended to be higher ($P \leq 0.08$) with HF compared to LF. Additional CO decreased recovery of total BCFA and *anteiso* odd-chain with HF treatments but increased recovery with LF treatments ($P \leq 0.09$, Diet × BCVFA). Dose recovery in total BCALD was higher ($P = 0.01$) with HF versus LF by 29.0% and recovery tended to be higher ($P = 0.07$) for total *iso* even-chain ALD with HF versus LF by 44.3%. Supplementation of CO with HF treatments decreased ^{13}C recovery in total BCALD whereas supplementation with LF increased ($P = 0.03$, Diet × BCVFA). The distribution of recovered dose in total BCFA and *anteiso* BCFA, which is the proportion of total ^{13}C recovered in bacteria pellets and reported in Chapter 4, decreased ($P < 0.10$, Diet × CO) when CO was supplemented in with HF but increased with LF.

Table 5.5. Dose recovery in bacterial lipids in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation.

	HF[1]		LF			Significance[2]		
Item	– CO	+ CO	– CO	+ CO	SEM	Diet	CO	Diet × CO
Total ^{13}C recovery in bacterial lipid C flow, µg/mg of ^{13}C dosed[3]								
Total lipid	23.0	18.7	14.1	15.2	1.3	< 0.01	0.30	0.02
BCFA[4]	17.7	13.8	10.3	10.9	1.7	0.01	0.25	0.03
iso even BCFA	4.59	3.02	2.44	1.72	0.61	0.08	0.12	0.42
anteiso BCFA	7.90	6.56	4.75	5.44	0.91	0.01	0.51	0.03
iso odd BCFA	5.30	4.32	3.04	3.68	0.44	0.06	0.76	0.10
BCALD[5]	5.29	4.97	3.71	4.25	0.77	0.01	0.83	0.03
iso even BCALD	1.88	1.61	1.22	1.20	0.20	0.07	0.37	0.42
anteiso BCALD	2.34	2.34	1.73	2.07	0.27	0.12	0.33	0.34
iso odd BCALD	1.10	1.07	0.72	0.93	0.15	0.14	0.41	0.32
Proportion of recovered dose in lipid C, %[6]								
Total lipid	14.7	13.4	16.4	15.6	2.2	0.38	0.66	0.92
BCFA	11.3	10.0	12.2	11.1	2.1	0.59	0.54	0.96
iso even BCFA	2.91	2.11	3.07	1.84	0.71	0.93	0.23	0.75
anteiso BCFA	4.97	4.61	5.75	5.66	1.01	0.30	0.78	0.87
iso odd BCFA	3.42	3.27	3.39	3.57	0.51	0.78	0.98	0.73
BCALD	3.36	3.42	4.22	4.49	0.60	0.11	0.72	0.82
iso even BCALD	1.19	1.07	1.47	1.32	0.23	0.26	0.52	0.94
anteiso BCALD	1.47	1.57	1.98	2.22	0.29	0.06	0.43	0.74
iso odd BCALD	0.707	0.781	0.771	0.953	0.13	0.28	0.25	0.59

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil).

[2] *P* values for main effect means for diet (HF vs LF), CO, interactions, the model also included a covariate (BCVFA mmol/d production) for the differences between diet for RDP and feed AA profile.

[3] The ^{13}C dose was provided as branched-chain VFA (5 mg/d of ^{13}C from each isobutyrate, 2-methylbutyrate, and isovalerate)

[4] Branched-chain fatty acids (BCFA).

[5] Branched-chain aldehydes (BCALD).

[6] Proportion of recovered dose was calculated by 100 × (mg ^{13}C recovered in bacterial lipid C flow/ mg ^{13}C recovered in bacterial total C flow).

Discussion

Biohydrogenation

Decreasing forage and increasing PUFA increased biohydrogenation when utilizing the calculation described by Tice et al. (1994), which is expressed as a percentage of unsaturated bonds in C18 FA that were that were saturated. With additional ground corn and corn oil, we expected that biohydrogenation extent and intermediates of the alternate *trans*-10 pathway would increase because of the increased 18:2 *cis*-9, *cis*-12 supply (Duckett et al., 2002; Zhang et al., 2019). In our study, pH was managed to be similar between diets, which often lower pH increases 18:1 *trans*-10 accumulation because of the combination of more starch and lower pH (Fuentes et al., 2009).

We hypothesized that BCVFA, which are growth factors for many important cellulolytic bacteria, would increase the index of biohydrogenation of PUFA but this did not occur. Additionally, butyrivibrios that biohydrogenate via the *trans*-11 pathway are characterized as including BCFA in their membrane structure and could use exogenous BCVFA rather than synthesize them; the stearate producers represented by *B. proteoclasticus* have particularly high BCFA concentrations (Hackmann and Firkins, 2015a). Previously, BCVFA and valerate supplementation did not increase biohydrogenation to 18:0 in vitro (Wu and Palmquist, 1991). A diet designed to depress milk fat increased 18:1 *trans*-10 and 18:2 *trans*-10, *cis*-12 percentages of total milk FA with isoacid supplementation (Lee et al., 2021). However, the isoacid treatment still alleviated milk fat depression and increased milk concentration of de novo fatty acids (Copelin et al., 2021). Milk fat concentration increased with 2-methylbutyrate and

isobutyrate supplementation in multiparous cows (Chapter 3). The supplementation of BCVFA increased de novo fatty acid synthesis in the mammary gland, as supported by increased expression of mammary FA synthesis genes expected by increased NDF degradation and greater acetate supply (Liu et al., 2018a). Although those authors suggested that isoacid supplementation would increase acetate supply as a mechanism for increased de novo synthesis of FA in the mammary gland, NDF degradability did not increase in Copelin et al. (2021), and acetate:propionate only numerically increased (Lee et al., 2021).

Feeding fat can increase accumulation of FA in lipid droplets of particulate-phase bacteria (Bauchart et al., 1990). Though bacterial 18:1 FA concentrations did increase with CO, the bacteria FA percentage of OM was not affected, potentially because the linoleic acid dose was measured to be only an additional 0.353 g/d with HF + CO versus HF – CO but was 0.799 g/d greater with LF + CO versus LF – CO. The FA concentration in the diets from Bauchart et al. (1990) ranged from 1.90 to 18.2% FA, compared to 2.69 to 4.01% in this study. We designed our diets to avoid unphysiologically excessive FA supply, which should have minimal depression of NDF degradability (Weld and Armentano, 2017). Therefore, to observe lipid droplet accumulation in solid adherent bacteria, supplemental FA supply probably would need to exceed amounts fed in this study. The increased FA concentration with decreased forage:concentrate in this study are more likely to be the result of prokaryotic profile changes, especially major shifts from Bacteroidetes to Proteobacteria populations previously described in Chapter 4.

Bacterial Lipids

In this study total ECFA in the bacterial profile did not change but shifted towards shorter chain ECFA with decreased forage:concentrate ratio. Lower chain length, decreased 18:0 or 14:0, and increased shorter even-chain FA, 12:0 or 16:0, have been reported before with lower forage:concentrate or decreased pH (Alves et al., 2013; Roman-Garcia et al., 2021c), which would decrease membrane fluidity (Or-Rashid et al., 2007). Decreasing forage:concentrate also had lower flow of 15:0 (Alves et al., 2013) and decreasing pH OCFA increased 15:0 FA in the bacterial FA profile (Roman-Garcia et al., 2021c). Other reports of forage:concentrate on OCFA have been mixed and it was theorized the varied results could be due to differing propionate supply, the primer for OCFA synthesis (Vlaeminck et al., 2006). It was expected that with decreasing forage in our study that OCFA would be greater in the FA profile as the diet shifted the microbiome towards a population that prefers to use OCFA for membrane homeostasis rather than BCFA (Kaneda, 1991). However, in our study 18:1 FA isomers replaced BCFA and OCFA in the bacterial FA profile in addition to decreased ECFA chain length was used to maintain membrane fluidity with decreased forage:concentrate.

Both pH and forage:concentrate ratio influenced bacterial FA profile, but decreasing pH did not change total BCFA concentration because even though *iso* even-chain and *iso* odd-chain FA decreased with low pH *anteiso* odd-chain FA increased (Roman-Garcia et al., 2021c). Alves et al. (2013) also reported increased *iso* FAME with increased forage (lucerne) but decreased *anteiso* FAME with decreased forage

(concentrate). They did not report pH in their results, but the lucerne diet likely had higher pH than the concentrate diet. In our study, decreasing forage decreased all BCFA in the bacterial FA profile. Additionally, the proportion of 15:0 *anteiso* in total odd branched-chain FA was negatively correlated to forage inclusion (Vlaeminck et al., 2006). Therefore, an increase in *anteiso* BCFA in bacteria probably is a specific adaptation to decreasing pH or to a shift in bacterial FA profile that was not replicated with only changing forage:concentrate.

Decreased forage:concentrate ratio decreased ECALD in bacterial ALD profile with our study, especially 16:0 ALD, and 16:0 DMA decreased in the rumen DMA profile reported by Alves et al. (2013) and Ventto et al. (2017). Both studies reported lower 13:0 and 15:0 DMA with decreased forage:concentrate and overall odd-chain DMA decreased. This was the opposite reported here because OCALD increased with lower forage:concentrate. In particular 17:0 ALD increased in our study which was replicated in Alves et al. (2013), this would increase the chain length of OCALD. In our study the shorter chain ECALD and longer chain OCALD replaced BCALD in the bacterial ALD profile to maintain membrane homeostasis.

Our results are generally consistent with increased BCALD in the ALD profile (or branched-chain DMA in the DMA profile) with increased forage:concentrate (Alves et al., 2013; Ventto et al., 2017). Only *anteiso* BCALD remained unchanged with increased forage inclusion in our study. Alves et al. (2013) also did not observe *anteiso* changing by forage:concentrate ratio, and the increase in total branched-chain DMA omasal flow was mainly due to an increase in 14:0 *iso*. Ventto et al. (2017) recorded increased branched-

chain DMA as a proportion of total DMA with increased forage:concentrate, which was due to increased *anteiso* and *iso* DMA. The BCALD (50.0 to 56.3% of total ALD) were much higher than their respective BCFA parent compounds (6.39 to 10.4% of total FA). Although DMA profiles ranged from 17.5 to 34% branched-chain DMA in previous reports (Alves et al., 2013; Ventto et al., 2017; Mannelli et al., 2018), authors did not evaluate lipid profiles of isolated bacteria. Because dietary FA would have diluted FA but not ALD, our results indicate that BCFA in phospholipids are even more preferentially converted to plasmalogens and subsequently measured as BCALD. Though increasing forage:concentrate did increase BCALD, the increase in our study (~ 8% increase) is much smaller than the difference reported (~ 49% increase) in Alves et al. (2013), who varied forage:concentrate more extensively than in our study and did not maintain ruminal pH to be similar between diets. Therefore, inclusion of BCALD in the structure with increasing forage:concentrate may also be a response to increased pH which will require a more fluid membrane.

The forage:concentrate influenced total BCFA and BCALD in our study, but CO supplementation only decreased *iso* even BCFA and BCALD in bacterial lipid profiles. Which indicates in our study supplementary oil specifically decreased isobutyrate incorporation into bacterial membranes. With soybean oil supplementation, total DMA flow did not change, but 15:0 *anteiso* DMA in the rumen DMA profile increased (Alves et al., 2013), whereas total DMA omasal flow decreased with sunflower oil (Ventto et al., 2017). The oil treatment from Alves et al. (2013), 10% DM, and Ventto et al. (2017), 5% DM, were much higher than the oil dose in our study, 3% DM. We expected that PUFA

supplementation would decrease total BCL in bacterial lipids due to the bacteriostatic effects of PUFA on cellulolytics and butyrivibrios, which were expected to incorporate more BCL in their membranes. However, the oil source, inclusion level, and or other environmental differences might also influence the BCL composition.

Despite greater availability of BCVFA primers with supplementation there were minimal differences in the total inclusion of BCL in bacterial membranes. Supplementation of BCVFA increased 18:1 isomers in the bacterial FA profile, which was consistent with increased effluent flow of 18:1 isomers, but decreased the percentages of ECFA and 16:0 in particular. Previously, Roman-Garcia et al. (2021c) reported decreased 13:0 *anteiso* and increased 14:0 *iso* with BCVFA supplementation so the total BCFA did not change. There were minimal differences in the ALD profile with supplemental BCVFA with lower forage:concentrate. Whereas with higher forage:concentrate BCVFA supplementation increased longer chain ALD and decreased shorter chain ALD for OCALD, *iso* even-chain ALD, and *anteiso* odd-chain ALD so the total sums in the ALD profile were not different.

The profile of bacterial FA and ALD had similar changes under the different dietary conditions, for example BCALD and BCFA percentage both increased with greater forage:concentrate and *iso* even BCFA and *iso* even BCALD both decreased with PUFA. However, there was clear preferential conversion of phospholipids containing BCFA, especially 14:0 *iso* and 15:0 *anteiso* BCFA, to plasmalogens (Alves et al., 2013; Ventto et al., 2017). Generally, the ALD profile shift towards a more fluid profile than would be expected if there was no preferential conversion of phospholipids to plasmalogens. Plasmalogens are more rigid than their phospholipid counterparts (Goldfine, 2010a);

therefore, inclusion of more BCALD and OCALD plus and shorter ECALD in the structure may be necessary under our conditions to maintain membrane fluidity.

Isotope Recovery

The recovery of ^{13}C in bacterial FA total C flow did not differ by pH (Roman-Garcia et al., 2021c), but this recovery increased with greater forage inclusion in our study. Presumably, fibrolytics were more likely to incorporate BCVFA primer into bacterial components (Vlaeminck et al., 2006). However, increased pH also increased the relative abundance of fibrolytic bacteria in the prokaryotic profile but not the incorporation of BCVFA into bacterial components (Roman-Garcia et al., 2021c). *Eubacterium cellulosolvens* 5494 increased membrane fluidity by increasing medium-chain and 18:1 FA compared with 18:0 when grown on a medium with cellulose versus glucose, cellobiose or fructose (Moon and Anderson, 2001). With PUFA supplementation, recovery decreased with in the high forage:concentrate, perhaps because the bacterial profile shifted towards a population that was more sensitive to PUFA.

Roman-Garcia et al. (2021c) used a shorter (60-m) column than ours (100-m) and reported notable isotope recovery in ECFA, primarily 16:0; however, our current results suggest that these conclusions were more likely a result of coelution of DMA with their identified FAME fractions. In the current study with the longer column, there was no significant enrichment except for that in BCL. There were no unidentified enriched FA, and in the DMA fraction there were unknown enriched peaks. Therefore, recovery in

anteiso lipids must come from ^{13}C dosed as 2-methylburyate, *iso* even from dosed isobutyrate, and *iso* odd from dosed isovalerate. These minor DMA peaks were not initially discovered until the DMA sample was injected with a higher volume on the column, particularly for the very small peaks eluting between 12:0 and 14:0 *iso* DMA. These were not identified in any other papers that analyzed DMA because those samples were from methylated rumen contents and likely these minor compounds were likely below detection limits.

The recovery in *iso* odd BCFA was greater than the recovery in *iso* even BCFA, but recovery in *iso* even BCALD was greater than the recovery in *iso* odd BCALD, indicating that there is preferential conversion of *iso* even BCFA in phospholipids to plasmalogens with *iso* even BCALD compared phospholipids with an *iso* odd BCFA. Just as in our study, Roman-Garcia et al. (2021c) also had greater ^{13}C recovery in *anteiso* BCFA than *iso* odd or *iso* even BCFA. In both studies, the dosed 2-methylbutyrate is a racemic mix; therefore, the requirement for 2-methylbutyrate for bacterial lipid synthesis apparently is even more significant than our isotope recoveries indicate because of stereo preference (Eibler et al., 2017).

Conclusion

Supplementation of BCVFA did not improve biohydrogenation of dietary FA or increase the total amount of BCFA or BCALD in bacterial membranes. Label was only recovered in BCFA and BCALD. Decreasing forage decreased the proportion of bacterial

lipids that were BCFA and BCALD that decreased the incorporation of ^{13}C from supplemented BCVFA. Corn oil decreased *iso* even BCL in both the FA and ALD profile, which indicated that isobutyrate was inhibited from incorporation into bacterial membrane structure with increasing PUFA. The greatest recovery of label was from *anteiso* BCFA and *anteiso* BCALD, indicating that 2-methylbutyrate was the BCVFA used the most as a primer for BCL synthesis. Whereas isovalerate (*iso* odd-chain) was incorporated more than isobutyrate (*iso* even-chain) for BCFA synthesis, isobutyrate was incorporated more in BCALD than isovalerate. Even though BCALD accounted for just ~ 6% of bacterial lipids, the recovery of isotope in BCALD was ~26% of the total dose in lipids due to the high percentage of ALD that were BCALD. Because BCFA and BCALD play an important role in the function and growth of bacteria, especially cellulolytics, BCVFA supplementation can support the rumen microbial consortium, increasing fiber degradation and efficiency of microbial protein synthesis.

Chapter 6. Effects of branched chain volatile fatty acids supplementation on protein metabolism and incorporation into microbial protein in dual flow cultures varying forage and corn oil concentration

Introduction

Fermentation of branched-chain AA (BCAA; Val, Ile, Leu) begins with the deamination or transamination to the corresponding α-ketoacids (Dehority et al., 1958; Kaneda, 1977; Annous et al., 1997). The α-ketoacids are rapidly decarboxylated to the acyl-CoA derivatives by branched-chain α-keto acid dehydrogenase using NAD^+ as an electron carrier in non-rumen model bacteria, but this enzyme is poorly represented in genomes of sequenced rumen bacteria (Roman-Garcia et al., 2021b). Instead, those authors described the likely role of ferredoxin as a cofactor for decarboxylation. Assuming ferredoxin is used as a cofactor for reductive carboxylation (Allison et al., 1984), bacteria such as the highly abundant *Prevotella ruminicola* could both decarboxylate α-ketoacids and reverse this process by reductively carboxylating BCVFA. The BCVFA-CoA derivatives can be converted to branched-chain VFA (BCVFA; isobutyrate, 2-methylbutyrate, isovalerate) by acyl-CoA hydrolase; in reverse, the BCVFA likely can be taken up and reactivated with a CoA transferase. Both uptake of preformed BCAA and recycling between BCVFA is important in mixed ruminal microbes (Atasoglu et al., 2004).

Thus, amylolytic bacteria can provide BCVFA for cellulolytics requiring them but also outcompete cellulolytics as starch increases and RDP becomes more limiting. Other amylolytic bacteria including *Streptococcus bovis*, *Selenomonas ruminantium,* and *Ruminobacter amylophilus*, and the hemicellulolytic/pectinolytic *Butyrivibrio fibrisolvens* do not produce BCVFA even when provided with BCAA (Allison, 1978; Stackebrandt and Hippe, 1986). Either these bacteria cannot transport BCAA or the bacteria lack the enzymes to catabolize BCAA. Stickland reactions convert BCAA to BCVFA, but their importance in the rumen is not well known or required (Hino and Russell, 1985). Regardless of the process, the formation of BCVFA leads to cross-feeding by fibrolytic bacteria that consume the BCVFA to develop an efficient consortium in the rumen (Morais and Mizrahi, 2019), and that efficiency should be decreased if BCVFA become insufficient. We therefore hypothesized that BCVFA supplementation would increase bacterial AA flow in both high forage diets (more cellulolytics) and low forage diets (cellulolytics have more competition for BCVFA). When polyunsaturated fatty acids (PUFA) are added, the competition by amylolytics might be further exacerbated because there is less forage on which lipids can adsorb and be biohydrogenated (Jenkins et al., 2008) and therefore they should be more toxic to the adherent cellulolytics.

Our objective was to investigate the usage of BCVFA supplementation on bacterial AA metabolism under varying dietary conditions in dual flow continuous cultures. We expected that supplemental BCVFA would increase bacterial AA outflow. We hypothesized that increased forage:concentrate would increase the incorporation of supplemental BCVFA into bacterial AA by increasing the abundance of cellulolytic

population that require BCVFA. Finally, supplementing PUFA in the diet was hypothesized to decrease BCVFA usage for bacterial AA synthesis because PUFA was assumed to inhibit cellulolytic bacteria.

Material and Methods

Experimental Design and Sample Collection

The experiment was a 2 × 2 × 2 factorial arrangement of 8 treatments of high (67:33) or low (33:67) forage:concentrate, without or with 3% corn oil (**CO**), and without or with dosed BCVFA supplementation (2.15 mmol/d each of isobutyrate, isovalerate, and 2-methylbutyrate). Dietary conditions are described in Chapter 4. Briefly, there were 8 anaerobic dual-flow continuous culture systems administered these 8 treatments in 4 periods in a randomized incomplete block (n = 4).

Background samples were taken on d 5 to measure the natural enrichment of ^{13}C in the dual-flow cultures. Samples were also taken during the collection period, which was d 9 through 12. After background sampling (d 5) was completed, [2-methyl-^{13}C]-2-methylbutyrate, [2,3-$^{13}C_2$, 2-methyl-^{13}C]-isobutyrate, and [2,3,4-$^{13}C_3$, 3-methyl-^{13}C]-isovalerate (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) replaced a portion of the unlabeled BCVFA dose through d 12 to provide equivalent to 5 mg/d ^{13}C from each BCVFA. The source of labeled 2-methylbutyrate is racemic. On d 9 to 12, effluent samples were collected and partitioned into respective aliquots, as described in Chapter 5.

Amino Acid Analysis and Computations

Feed samples, lyophilized effluent, and bacterial pellets were sent to Cumberland Valley Analytical Services (Waynesboro, PA) for AA quantification. The samples underwent separate hydrolysis procedures for Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile, Leu, Tyr, Phe, His, Lys, Arg (AOAC, 1997; method 994.12, Gehrke et al., 1987); Cys, Met, (AOAC, 2006; method 982.30 E(a,b,c)), which included performic acid protection against sulfur AA destruction. Both hydrolysates were analyzed by ion exchange chromatography (AOAC, 1997; method 994.12). Alkaline hydrolysis was performed with barium hydroxide and 5-methyltryptophan as the internal standard (AOAC, 2006; method 988.15), and Trp was analyzed with chromatography with fluorescence detection (Landry and Delhaye, 1992). Correction factors from Lapierre et al. (2019) adjusted for differential recoveries of AA with 24-h hydrolysis, which was necessary for unbiased ^{13}C recovery calculations. Lyophilized effluent and bacteria pellets were also analyzed for N (AOAC, 2006; method 990.03). The results of feed AA analysis are showing in Table 6.1.

Table 6.1 Amino acid composition of the diets varying in forage and corn oil supplementation.

Item	HF – CO[1]	HF + CO	LF – CO	LF + CO
AA Profile, g/100 g total AA				
Cys	0.589	0.565	0.800	0.955
Met	1.40	1.41	1.25	1.25
Asp	11.0	11.1	10.4	10.4
Thr	4.41	4.33	4.10	4.02
Ser	5.30	5.25	5.47	4.75
Glu	14.7	14.7	16.7	17.4
Pro	7.28	7.28	7.15	7.13
Gly	5.45	5.35	5.16	5.11
Ala	6.12	6.12	5.80	5.78
Val	6.23	6.22	5.73	5.70
Ile	5.05	5.03	4.96	4.74
Leu	8.86	8.86	9.09	9.12
Tyr	3.60	3.58	3.74	3.71
Phe	5.54	5.55	5.15	5.26
His	2.15	2.15	2.33	2.36
Lys	5.04	5.05	4.95	5.09
Arg	5.58	5.72	5.88	5.93
Trp	1.77	1.73	1.41	1.30

[1] Treatments are high forage (HF, 67% forage) or low forage (LF, 33% forage) and – CO (no additional supplemented fat) or + CO (3% DM supplemented fat as corn oil).

The isotope ratios of bacterial AA were determined after acid hydrolysis followed by N-acetyl isopropyl derivatization (Styring et al., 2012) on a GC (Trace 1300; Thermo Fisher Scientific, Waltham, MA) equipped with a Isotope ratio mass spectrometry. For the separation of AA with GC, a DB-1301 column (Agilent, Santa Clara, CA; 60 m × 0.25 mm × 1.0 µm) and a 5-m deactivated guard column were used. A 2-µL sample was injected, splitless, at an inlet temperature of 225ºC. The carrier gas was He at 1.2 mL/min. The initial oven temperature was 70ºC was held for 2 min before a 15ºC/min increase to 140ºC, which

was and held for 4 min. The temperature was then increased at 12ºC/min to 240ºC and held for 5 min. The final temperature increase was 8ºC/min to 255ºC and held for 35 min. The eluate was then combusted through a combustion reactor (NiO and CuO at 1,000°C) and introduced into isotope-ratio MS (Delta V Advantage; Thermo Fisher Scientific) for ^{13}C enrichment of compounds m/z 44, 45, and 46). Isotope-ratio MS was calibrated for ^{13}C enrichment using standards with known ^{13}C enrichment (IAEA-600; Vienna, Austria; n18M; Indiana University, Bloomington, Indiana; USGS71 and USGS72; Reston Stable Isotope Laboratory, Reston, Virginia).

Total N and bacterial N flows are presented in Chapter 4. Respective N flows were converted to AA flows based on the equation N (g/d) × % AA of sample/ % N of sample. Nonbacterial AA was total AA flow – bacterial AA flow. The AA-N flows were calculated by multiplying AA flows by the % N of each AA. The flows Asp/Asn and Glu/Gln were multiplied by the average % N to account for the loss of 1 N atom during hydrolysis of Asn to Aps and Gln to Glu. The N-acetyl isopropyl derivatization adds a propyl group (3 C) per carboxyl group and an acetyl group (2 C) per amino group in the AA structure (i.e., Leu has 6 C, but derivatized Leu has 11 C). Therefore, ^{13}C enrichment of AA (corrected for background ^{13}C) is diluted by the unlabeled C from derivatization. The ^{13}C in bacterial derivatized AA was calculated as its atom percent excess ^{13}C divided by 100 and multiplied by C flow of the derivatized AA; the total ^{13}C recovered in each AA was then divided by total ^{13}C dosed. The total ^{13}C recovered in the bacteria C outflows was reported in Chapter 4. This recovered ^{13}C was partitioned into BCAA as reported herein, the total ^{13}C recovered as lipids (Chapter 5), and the remainder not in either fraction.

Statistical Analysis

Bacterial AA, total AA, and nonbacterial AA flow and profile measurements were analyzed using PROC MIXED in SAS 9.4 (SAS Institute Inc.) according to the model described in Chapter 4. Briefly a mixed model had random effects of period and fermenter and fixed effects of diet, CO, BCVFA, and their interactions. Recovery of ^{13}C dose was analyzed with the same model but had an additional covariate because LF and HF differed in calculated RDP and therefore basal BCVFA production (Chapter 4).

Results

Bacterial AA flow and profile

The total AA percentage of OM and total AA-N percentage of total N in bacteria both were greater ($P < 0.01$, Table 6.2) by 15.0% and 4.0%, respectively, with LF versus HF. However, supplementation of BCVFA decreased total AA in OM in HF treatments but increased it in LF treatments ($P = 0.09$, Diet × BCVFA). The total AA-N decreased by 2.25% of total N with BCVFA supplementation to HF treatments but increased by 3.59% of total N with BCVFA in LF treatments ($P = 0.02$, Diet × BCVFA). With CO supplementation, AA-N as a percent of total N decreased ($P = 0.10$, Diet × CO) in both diets, but the decrease was more with LF than HF. Efficiency of bacterial AA-N per kg truly degraded OM or g truly degraded N both increased ($P < 0.02$) with BCVFA supplementation by 7.14% and 7.28%, respectively.

Total bacterial AA flow increased when BCVFA was supplemented with HF – CO by 16.6%, LF – CO by 6.66%, and LF + CO by 12.4%, but bacterial AA flow decreased by 1.5% when BCVFA was supplemented with the HF + CO diet ($P = 0.07$, Diet × CO × BCVFA; Table 3). Total bacterial BCAA flow was greater ($P < 0.01$) by 9.1% with LF versus HF, bacterial BCAA flow tended to decrease ($P = 0.08$) by 5.1% with CO versus without CO, and it increased ($P < 0.01$) by 10.7% with BCVFA versus without BCVFA supplementation (Interactions were $P > 0.11$). The remaining bacterial AA flows are presented in Table F.1.

Total BCAA in the total AA was higher with LF versus HF due to an increase ($P < 0.01$) of Val and Leu, and a numerical increase in Ile (Table 6.2). The percentages of Cys, Pro, Gly, and Trp in total AA tended to be lower ($P \leq 0.07$) with LF versus HF, whereas Lys was greater ($P = 0.01$) with LF versus HF. When BCVFA were supplemented to HF diets, Pro percentage of total AA did not change, but Pro decreased when BCVFA were supplemented to LF ($P = 0.10$, Diet × BCVFA). When BCVFA were supplemented to HF diets, Gly and Ala increased, whereas the profile of these AA decreased when BCVFA were supplemented to LF treatments ($P < 0.07$, Diet × BCVFA). In HF treatments, BCVFA decreased Arg profile, but BCVFA increased Arg in LF treatments ($P = 0.03$, Diet × BCVFA). Supplemental BCVFA increased Met and Cys without CO, but BCVFA decreased Met and Cys profiles with CO supplementation ($P < 0.04$, CO × BCVFA). Supplemental BCVFA increased ($P = 0.02$) total BCAA by 1.99% of total AA. This change was a result of trends or numerical increases in Val (1.95%, $P = 0.08$), Ile (2.01%, $P = 0.06$), and Leu (1.99%, $P = 0.12$).

Table 6.2. Bacterial AA concentration, efficiency of bacterial amino acid N (AA-N) synthesis, AA flow, and AA profile in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation.

	HF[1]				LF					Significance[2]			
	– CO		+ CO		– CO		+ CO		SEM				Diet ×
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA	CO × BCVFA
AA, % OM[3]	59.7	57.0	60.3	59.0	68.3	69.9	63.4	69.8	3.5	< 0.01	0.71	0.55	0.60
AA-N, % total N[3, 4]	81.6	77.3	78.4	79.1	82.4	84.3	79.3	83.2	1.3	< 0.01	0.15	0.55	0.43
Efficiency of microbial AA-N synthesis, g/degraded nutrient													
Bacterial AA-N/kg OM	18.5	20.0	20.0	19.7	18.8	20.5	17.4	19.7	0.8	0.44	0.69	0.02	0.27
Bacterial AA-N/g N	0.623	0.681	0.646	0.640	0.646	0.704	0.595	0.669	0.029	0.69	0.12	0.01	0.21
Bacterial Flow, g/d													
Total AA	6.31	7.36	6.59	6.49	7.12	7.60	6.57	7.38	0.30	0.02	0.09	< 0.01	0.07
Total BCAA[5]	1.27	1.48	1.30	1.32	1.44	1.57	1.32	1.53	0.07	< 0.01	0.08	< 0.01	0.11
Bacterial AA Profile, g/100 g total AA													
Cys[4]	1.10	1.16	1.19	1.14	1.00	1.03	1.10	0.91	0.06	< 0.01	0.75	0.25	0.40
Met[4]	2.14	2.30	2.51	2.33	2.11	2.34	2.48	2.08	0.09	0.24	0.03	0.40	0.21
Asp	12.1	12.1	12.1	12.2	12.1	12.0	12.1	12.0	0.1	0.36	> 0.99	0.72	0.55
Thr	5.55	5.55	5.60	5.63	5.63	5.50	5.50	5.60	0.09	0.41	0.99	0.72	0.65
Ser	4.83	4.85	4.86	4.83	4.91	4.88	4.86	4.82	0.07	0.60	0.60	0.63	0.85
Glu	12.8	13.0	12.8	12.8	12.8	12.8	12.8	12.8	0.1	0.48	0.62	0.53	0.35
Pro[3]	3.59	3.71	3.68	3.66	3.75	3.49	3.50	3.48	0.09	0.07	0.32	0.43	0.10
Gly[3]	5.39	5.52	5.40	5.43	5.42	5.34	5.41	5.34	0.03	0.01	0.31	0.97	0.14
Ala[3]	6.91	7.05	6.89	6.88	6.96	6.82	6.94	6.87	0.08	0.41	0.39	0.62	0.26

continued

Table 6.2 continued

Item	HF[1]				LF				SEM	Significance[2]			
	– CO		+ CO		– CO		+ CO						Diet ×
	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA	CO × BCVFA
Val	6.64	6.46	6.46	6.70	6.60	6.78	6.55	6.80	0.11	0.09	0.94	0.08	0.22
Ile	5.87	5.82	5.76	5.97	5.88	5.98	5.81	6.03	0.09	0.23	0.93	0.06	0.56
Leu	7.65	7.73	7.56	7.64	7.71	7.95	7.69	7.90	0.15	0.10	0.49	0.12	0.94
Tyr	4.87	4.86	4.79	4.84	4.84	4.83	4.82	4.80	0.06	0.58	0.30	0.98	0.62
Phe	4.81	4.86	4.82	4.85	4.85	4.85	4.82	4.81	0.05	0.88	0.33	0.39	0.79
His	1.68	1.67	1.67	1.69	1.69	1.70	1.68	1.68	0.03	0.36	0.70	0.78	0.35
Lys	7.40	7.48	7.44	7.44	7.67	7.57	7.73	7.70	0.07	< 0.01	0.21	0.71	0.31
Arg[3]	5.01	4.36	4.88	4.36	4.47	4.63	4.65	4.89	0.24	0.96	0.65	0.26	0.96
Trp	1.58	1.61	1.62	1.62	1.57	1.52	1.61	1.52	0.07	0.06	0.39	0.31	0.80

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] *P* values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] Interaction of Diet and BCVFA supplementation ($P \leq 0.10$).

[4] Interaction of CO and BCVFA supplementation ($P \leq 0.10$).

[5] Branched-chain AA (BCAA) is the sum of Val, Ile, and Leu.

Isotope recovery in bacterial AA

Only bacterial BCAA were enriched in our study (Table 6.3). Recovery of dose was greater ($P \leq 0.02$) by 45.8% in total BCAA, by 55.3% in Val, by 53.9% in Ile, and by 39.6% in Leu with HF diets compared to LF diets. The proportion of ^{13}C in total bacteria C that was recovered in either bacterial AA C flow (65.2%), bacterial lipid C flow (14.0%), or not recovered in those sinks (other, 20.6%) was not influenced by any main effects or interactions. The distribution of recovered ^{13}C in individual fatty ands and aldehydes are reported in Chapter 5.

Table 6.3. Dose recovery in bacterial AA in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation.

	HF[1]		LF			Significance[2]		
Item	– CO	+ CO	– CO	+ CO	SEM	Diet	CO	Diet × CO
Total ^{13}C recovery in bacterial AA C flow, µg/mg of ^{13}C dosed[3]								
BCAA[4]	104	94.3	68.8	58.0	4.4	0.01	0.19	0.79
Val	23.2	21.5	15.1	12.6	1.3	0.02	0.22	0.69
Ile	29.2	26.9	19.0	16.3	1.2	0.01	0.23	0.77
Leu	51.2	45.7	34.9	29.2	2.2	0.01	0.17	0.97
Distribution of ^{13}C in bacterial outflow, %[5]								
BCAA	65.5	67.9	67.9	59.7	6.4	0.48	0.70	0.50
BCL[6]	14.7	13.4	12.5	15.6	1.0	0.38	0.66	0.92
Other[7]	19.9	18.7	19.2	24.8	3.2	0.45	0.52	0.37

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil).

[2] *P* values for main effect means for diet (HF vs LF), CO, interactions, the model also included a covariate (BCVFA mmol/d production) for the differences between diet for RDP and feed AA profile.

[3] The ^{13}C dose was provided as branched-chain VFA (5 mg/d ^{13}C from each isobutyrate, 2-methylbutyrate, and isovalerate)

[4] Branched-chain amino acids (BCAA) is the sum of Val, Ile, and Leu

[5] Proportion of recovered dose was calculated by 100 × (mg ^{13}C recovered in each fraction/ total mg ^{13}C recovered in bacterial C outflow).

[6] Branched-chain lipids (BCL) is the sum of *iso* even, *anteiso* odd, and *iso* odd fatty acids and aldehydes reported in Chapter 5.

[7] Other is the balance of ^{13}C recovered in bacterial 13C outflow that is not represented by BCAA or BCL.

Total AA flow and profile

Supplementation of BCVFA increased ($P \leq 0.01$) effluent AA concentration on a DM basis from 12.6% without BCVFA to 14.1% with BCVFA (SE = 0.8%, Table F.2). The effluent flow of total AA was 12.6 g/d with HF diets, which tended to be greater ($P = 0.07$) than 12.0 g/d with LF diets (SE = 0.1). Supplemental BCVFA increased ($P < 0.01$) total AA flow from 11.6 g/d without BCVFA to 13.1 g/d with BCVFA (SE = 0.9). The flow of total BCAA also increased ($P < 0.01$) from 2.48 g/d without BCVFA to 2.80 with BCVFA (SE = 0.13). Most of the flows of individual AA were greater ($P \leq 0.01$) when BCVFA were supplemented (Table F.2). The main effect of BCVFA on the relative ratio of the AA flows with BCVFA/without BCVFA are presented in Figure 6.1 for clarity. The greatest relative ratio was Ala (1.21) and Pro (1.17), whereas the remaining relative ratios ranged from 1.10 to 1.15. The relative ratio of Trp was only 1.05, and the flow of Trp did not increase with BCVFA supplementation ($P = 0.11$, Table F.2). The relative ratios of Cys, Met, and Arg are not presented in Figure 1 because there were interactions between BCVFA and other main effects (Table F.2). The flow of Cys increased when BCVFA was supplemented with HF diets but decreased with LF diets ($P = 0.07$, Diet × BCVFA). Both Met and Arg flow increased when BCVFA was supplemented without CO, but Met flow decreased when BCVFA was supplemented with CO whereas Arg flow still increased but to a lesser extent ($P \leq 0.09$, CO × BCVFA). Additional CO in the LF diet decreased Met flow, but there was no difference in flow with HF ($P = 0.09$, Diet × CO).

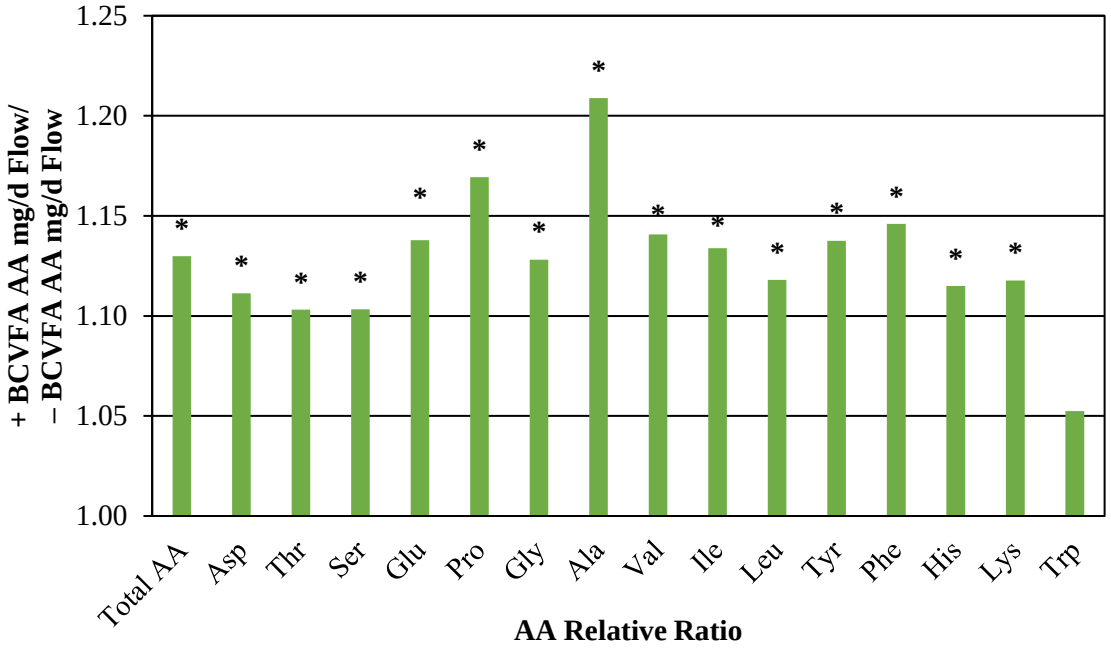

Figure 6.1. Relative ratio of effluent AA flows (mg/d) of continuous cultures with branched-chain VFA supplementation/effluent flow of AA from continuous cultures without BCVFA supplementation. Bars marked with * indicate $P \leq 0.01$ difference between the main effect means of with vs without BCVFA on AA flow.

Nonbacterial AA flow

The flow of nonbacterial total AA, total BCAA, Thr, Ser, Pro, Gly, Ala, Val, Ile, Phe, His, and Lys all experienced a 3-way interaction ($P < 0.10$, Diet × CO × BCVFA; Table 6.4). Supplementation of BCVFA increased the flows mainly with the HF + CO treatment and to a lesser degree with HF + CO and LF – CO diets, whereas BCVFA supplementation with LF + CO usually decreased nonbacterial AA flows. Supplementation of BCVFA increased nonbacterial Cys and Arg flows with HF diets but not LF diets ($P < 0.09$, Diet × BCVFA). The flows of nonbacterial Met, Asp, Leu, and Trp was lower ($P < 0.03$) with LF diets when compared to HF diets. Supplementation of BCVFA tended to

increase ($P = 0.09$) nonbacterial Met flow and increased ($P \leq 0.05$) flows of nonbacterial Glu, Leu, and Tyr.

Table 6.4. Flow of nonbacterial AA in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation.

	HF[1]				LF				SEM	Significance[2]			
	– CO		+ CO		– CO		+ CO						
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA	Diet × CO × BCVFA
Flow of nonbacterial AA, g/d[3]													
Total AA	5.35	6.11	5.21	7.06	4.26	5.72	4.80	4.50	0.86	0.01	0.92	0.02	0.06
Total BCAA[4]	1.22	1.38	1.21	1.59	1.03	1.25	1.14	1.09	0.14	0.01	0.58	0.03	0.10
Cys[5]	0.0527	0.0868	0.0524	0.0840	0.0360	0.0531	0.0613	0.0348	0.0162	0.05	0.93	0.20	0.34
Met	0.0730	0.0883	0.0718	0.0913	0.0459	0.0547	0.0401	0.0394	0.0148	< 0.01	0.43	0.09	0.58
Asp	0.462	0.511	0.466	0.610	0.338	0.466	0.413	0.377	0.103	0.03	0.63	0.14	0.18
Thr	0.237	0.252	0.220	0.287	0.167	0.242	0.210	0.179	0.046	0.03	0.99	0.13	0.07
Ser	0.297	0.304	0.265	0.336	0.230	0.301	0.243	0.235	0.048	0.03	0.52	0.11	0.10
Glu	0.740	0.862	0.727	0.970	0.675	0.893	0.745	0.734	0.133	0.24	0.98	0.01	0.11
Pro	0.355	0.401	0.354	0.468	0.285	0.427	0.326	0.349	0.059	0.07	0.78	0.00	0.07
Gly	0.333	0.374	0.309	0.421	0.271	0.347	0.282	0.271	0.049	< 0.01	0.60	0.01	0.06
Ala	0.357	0.419	0.332	0.607	0.295	0.515	0.326	0.281	0.107	0.20	0.86	0.04	0.05
Val[5]	0.294	0.364	0.287	0.422	0.248	0.317	0.284	0.228	0.056	< 0.01	0.97	0.02	0.04
Ile	0.257	0.304	0.275	0.363	0.206	0.276	0.257	0.228	0.043	0.01	0.34	0.04	0.10
Leu	0.667	0.714	0.651	0.805	0.571	0.656	0.603	0.633	0.058	0.02	0.57	0.05	0.29
Tyr	0.150	0.186	0.153	0.235	0.126	0.173	0.162	0.148	0.038	0.13	0.39	0.05	0.16
Phe	0.287	0.339	0.273	0.379	0.207	0.282	0.242	0.230	0.052	< 0.01	0.90	0.01	0.06
His	0.258	0.258	0.226	0.325	0.138	0.237	0.192	0.163	0.059	0.01	0.89	0.19	0.08
Lys	0.131	0.145	0.121	0.154	0.110	0.137	0.116	0.110	0.014	0.01	0.44	0.03	0.07
Arg[5]	0.284	0.386	0.305	0.374	0.228	0.256	0.222	0.176	0.041	< 0.01	0.33	0.06	0.60
Trp	0.119	0.116	0.123	0.135	0.084	0.083	0.077	0.084	0.016	< 0.01	0.54	0.60	0.80

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] P values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] Nonbacterial AA was calculated as Effluent AA flow – Bacterial AA flow.

[4] Branched-chain AA (BCAA) is the sum of Val, Ile, and Leu.

[5] Interaction of Diet and BCVFA supplementation has $0.03 \leq P \leq 0.09$.

Discussion

BCVFA usage by bacteria

Much more isotope was recovered in bacterial BCAA than bacterial lipids reported in Chapter 5 because the bacteria were about 73.7% AA but only 9.98% FA and 0.57% fatty aldehyde on OM bases. Therefore, recovery of ^{13}C is expected to be much greater in bacterial protein, especially because BCAA were > 20% of total AA in our study. Approximately 79.3% of the recovered dose in bacteria C flow was distributed in bacterial AA or lipids. The source of the 20.7% of recovered ^{13}C that was not recovered in lipids or AA is unknown. Allison and Bryant (1963) noted that nucleic acids accounted for approximately 2% of total radioactivity, compared to 17 to 25% in lipids and 70 to 79% in protein, of rumen contents incubated with ^{14}C-labeled isovalerate and isobutyrate. However, the mechanism is not clear how nucleic acids would be labeled. There could be alternate pathways of Leu (Monticello and Costilow, 1982; Atasoglu et al., 2004) and therefore isovalerate, and some Ile is synthesized from Thr (Kaiser and Heinrichs, 2018). However, the lack of ^{13}C recovery in the major FA other than BCFA (Chapter 5) argues against ^{13}C-BCVFA metabolism to central metabolites as a major route. Because pantothenic acid is synthesized from the ketoacid of Ile, some isobutyrate is likely converted to pantothenic acid (Kaiser and Heinrichs, 2018). However, this source would contribute and unmeaningful proportion to the unrecovered ^{13}C pool. Therefore, the remaining ^{13}C could be in intermediates of lipid or AA synthesis that are not recovered by our analytical methods and other sinks not analyzed in this study.

Even though flow of bacterial BCAA was greater with decreasing forage, the isotope recovery decreased. The prokaryotic profile shifted towards a population that requires BCVFA primers, as described in Chapter 3, with greater forage inclusion in the diet. Recovery of the label was the greatest in Leu, which was the BCAA highest in the bacterial AA profile. The recovery in Ile was the next highest. However, 2-methylbutrate in this study was dosed in a racemic mix, and our recovery in Ile therefore could be considered up to 32.6 µg/mg to 57.2 µg/mg of total ^{13}C dosed instead of 16.3 µg/mg to 28.6 µg/mg, as justified by Robinson and Allison (1969). Recovery in Val was lower than expected, especially when considering that it was the next greatest BCAA in bacterial AA profile after Leu. When isobutyrate was supplemented with labeled glucose *Bacteroides fragilis* and *Prevotella ruminicola*, which does not require BCVFA primers, the radioactivity of Val did not decrease; in contrast, when isovalerate or 2-methylbutyrate were provided, radioactivity of Leu or Ile decreased (Allison et al., 1984). Potentially, bacteria that do not require BCVFA precursors still benefit from isovalerate and 2-methylbutyrate supplementation and spare C, but isobutyrate was less beneficial for sparing energy for Val biosynthesis.

Isotope recovery in both bacterial Ile in the present study and *anteiso* odd-chain lipids was high (Chapter 5). Clearly, 2-methybutyrate must have been prioritized for lipids, especially because 2-methylbutyrate was racemic. However, only providing additional 2-methylbutyrate might inhibit rumen bacteria growth because Ile at low concentrations is inhibitory (Kajikawa et al., 2002). Additionally, as discussed by Roman-Garcia et al. (2021a), supplementation of an *iso* fatty acid precursor maybe necessary for eliciting the

benefits of BCVFA in a rumen. In our study, with decreasing forage:concentrate ratio decreased recovery of ^{13}C in Leu by approximately 40%, whereas recovery in both Val and Ile decreased by more than 54%. Thus, decreasing forage diet mainly decreased isobutyrate and 2-methylbutyrate incorporation into bacterial Val and Ile, respectively, rather than isovalerate into Leu. Even though ^{13}C recovery in Val was the lowest, phospholipids with an *iso* even branched-chain fatty acid were preferentially reduced to form plasmalogens (Chapter 5), indicative that benefits of isobutyrate supplementation maybe specific to bacterial membrane structure rather than bacterial protein. Previously discussed in Chapter 3 and Roman-Garcia et al. (2021a), the benefit of isovalerate supplementation may be limited because of the high Leu content already available in commercial diets. There was no recovery of label outside of BCAA in our study. When labeled BCVFA or BCAA were dosed, little label was recovered in other AA besides the corresponding BCAA (Allison et al., 1962a; Allison and Bryant, 1963; Robinson and Allison, 1969). There was also no isotope recovery outside of branched-chain lipids reported in Chapter 5. Our results indicate that BCVFA are being utilized as precursors for their specific BCAA or branched-chain lipid. Whereas BCVFA not utilized by rumen bacteria are mainly either absorbed or flow out of the rumen.

AA supply

Previously, we hypothesized that many of the benefits of BCVFA supplementation resulted from increased fiber degradation and microbial protein synthesis, but BCVFA in our study also specifically increased BCAA in bacterial AA profile and increased bacterial BCAA effluent flow. Increased metabolizable protein supply resulting from with increased

AA flow would improve milk production, especially milk protein yield (e.g. Nichols et al, 2019; Rius et al, 2010). The increased in BCAA supply can also have a post ruminal influence on protein and fat metabolism in various tissues in the dairy cow. In bovine mammary cells, mechanistic target of rapamycin (mTOR) phosphorylation, which partial mediates milk protein synthesis, increased with Leu and Ile (Kim, 2009; Appuhamy et al., 2012). Removal of BCAA in an infusion treatment deactivated mTOR complex 1 and protein yield decreased when compared to the total essential amino acid infusion (Doelman et al., 2015). Rapamycin also regulates genes related to milk fat synthesis (Bionaz et al., 2020). Previously demonstrated supplemental BCAA decreased hyperketonemia incidence in transition Holstein cows but did not increase milk production (Leal Yepes et al., 2019). In a following study, liver triglycerides were measured and decreased with BCAA supplementation (Leal Yepes et al., 2021). Therefore, BCVFA supplementation can have post ruminal influences on AA and fatty acid metabolism that still require further researched.

Previously, supplementation of 2-methylbutyrate increased CP effective degradability in situ (Wang et al., 2018), whereas supplementation of isobutyrate (Wang et al., 2015) and isovalerate (Liu et al., 2009a) decreased CP effective degradability in situ. End-product regulation of peptidases and deaminases has been suggested before (Bach, 2005) and the Prevotella species are major contributors to proteolysis in the rumen (Hartinger et al., 2018) and within our vessels. Previously, Griswold et al. (1996) demonstrated that several Prevotella strains decreased proteolytic activity with the supplementation of peptides. Most Prevotella, amongst many other rumen gram-negative

bacteria, have annotated leucine-responsive regulatory protein (Lrp) identified as K03719 in KEGG (Kanehisa et al., 2012; Seshadri et al., 2018). Higher [BCAA] increased binding of Leu to Lrp which increases AA transportation and biosynthesis whereas AA catabolism decreases (Calvo and Matthews, 1994). Additionally, the transcription regulator CodY (K03706) was widely annotated in gram-positive bacteria in the Hungate 1000 collection. Repression of CodY DNA-binding activity, with greater [BCAA] especially Ile, decreases expression of AA transportation and biosynthesis genes (Guédon et al., 2001; Ratnayake-Lecamwasam et al., 2001). The increased BCAA in bacterial AA profile could indicate supplementation of BCVFA has the potential to influence CodY and Lrp regulation of AA metabolism. Thus, the importance of BCAA in bacterial growth and AA metabolism seems highly likely and warrants future experimental confirmation.

Conclusion

Increasing forage increased the incorporation of BCVFA into bacterial protein, but the there was no appreciable label recovered outside of BCAA in this study. Previous research with BCVFA supplementation indicated that many of the benefits were due to increased NDF degradation and increased bacterial N flow. However, this study indicates that increased total AA flow and BCAA flow is an additional benefit that would increase metabolizable AA and result in greater milk production or production efficiency in ruminants.

Chapter 7. Conclusions

Rumen optimal function depends on a diverse microbial population and their interactions. Cross feeding of fermentation products can be a source of limited substrates such as branched-chain volatile fatty acids (BCVFA; isobutyrate, 2-methylbutyrate, and isovalerate). The BCVFA are produced from catabolism of branched-chain amino acids (BCAA; Val, Ile, and Leu) and are metabolized by cellulolytic bacteria that cannot uptake or degrade their own BCAA and require BCVFA for synthesis of BCAA or branched-chain fatty acids (BCFA; *iso* even-chain, *anteiso* odd-chain, iso odd-chain). Some of the BCFA on the sn-1 position of the phospholipid are converted to vinyl ethers in plasmalogens; these derivatives are recovered during methylation procedures as branched-chain aldehydes (BCALD). Branched-chain lipid structures are important to maintain membrane homeostasis and are highly enriched in cellulolytics. Cellulolytic bacteria are primary colonizers in the rumen ecosystem and help to expose more substrate or provide oligosaccharides from fibrolysis to support a consortium of secondary colonizers. In order to optimize ruminants as upcyclers of human-inedible biomass, efficient fiber degradation from a balanced consortium is necessary.

Our previous in vitro work indicated the greatest need was for 2-methylbutyrate, but further in vivo research was necessary. We tested different combinations of BCVFA

and valerate in Chapter 3 to determine an optimal blend. Though DMI and milk yield did not change with supplementation of isobutyrate and 2-methylbutyrate, both fat yield and feed efficiency, measured as net energy of lactation/DMI, increased (significance vs control was achieved in 2 of the 4 biweekly measurements). Feed efficiency also increased with supplementation of isobutyrate, 2-methylbutyrate, isovalerate, and valerate; however, results were similar to isobutyrate and 2-methylbutyrate and not different from control. The improvement was not the result of change in body weight, average daily gain, or body condition score. The production study suggested that isobutyrate in addition to 2-methybutyrate was able to improve feed efficiency and was a more economical treatment than supplementing all BCVFA and valerate in commercial herds. Especially, in diets that include a lot of Leu, which would provide sufficient isovalerate in the rumen, isovalerate probably does not need to be supplemented.

To investigate the influence of dietary conditions on BCVFA benefits and usage, dual flow continuous cultures were treated with diets varying in forage:concentrate and polyunsaturated fatty acids (PUFA). Cultures were either treated with control buffer or supplemented with BCVFA, and each individual ^{13}C-labeled BCVFA was provided with the BCVFA treatment. In Chapter 4, recovery of dosed ^{13}C was higher with greater forage:concentrate. Higher forage:concentrate shifted the prokaryotic profile towards a population that required BCVFA for growth and increased the incorporation of BCVFA into bacterial structures. However, neutral detergent fiber degradability and efficiency of bacterial N synthesis both increased with BCVFA supplementation under all dietary

conditions. Results indicate that BCVFA supplementation could benefit ruminants under a wide range of diet conditions, although individual BCVFA were not differentiated.

The usage of BCVFA for bacterial lipid synthesis was studied in Chapter 5 to help differentiate needs for individual BCVFA. Fatty acids (FA) in bacterial phospholipids were analyzed separately from aldehydes (ALD) from bacterial plasmalogens. With higher forage inclusion, there was a greater percentage of BCFA and BCALD in the bacterial FA and ALD profile. With all dietary conditions, over 50% of bacterial ALD were BCALD. Recovery of ^{13}C in bacterial lipid C was also greater with higher forage:concentrate ratio. Though ALD were only 6% of total ^{13}C recovered in lipids, recovery in ALD was 26.0% of that ^{13}C recovered in bacterial lipids. There was no appreciable label recovered outside of BCFA or BCALD. Previously reported enrichment from ^{13}C-labeled BCVFA recovered outside of BCFA was due to coelution of BCALD on the shorter column (Roman-Garcia et al., 2021c). Corn oil only decreased the percentage of *iso* even-chain lipids (from isobutyrate elongation) in the bacterial FA and ALD profiles. Recovery in *anteiso* odd-chain lipids was the highest, supporting previous reports that 2-methylbutyrate was especially important for rumen bacteria. In contrast, phospholipids with *iso* even-chain FA were preferentially converted to plasmalogens. Our results indicated that conversion of phospholipids to plasmalogen occurred more with phospholipids with more fluid acyl chains, especially BCFA. How the conversion is regulated and the role of plasmalogens in bacterial membranes still requires further study.

The usage of BCVFA for BCAA synthesis was investigated in Chapter 6. Again greater forage:concentrate increased dose recovery in bacterial amino acid (AA) C flow.

The most ^{13}C was recovered in Leu, followed by Ile, with Val being the lowest. No appreciable label was recovery outside of BCAA. The dosed 2-methylbutyrate used in our study was a racemic mix; therefore, 2-methylbutyrate as a primer is probably more important than the recovery values indicate. Overall, BCVFA were incorporated into their respective end-products based on expected metabolic schemes. The source of the ^{13}C in nonbacterial effluent is likely from outflow of dose, whereas the recovery of ^{13}C in bacteria but outside of AA and lipids is unknown. Because fermenters allow only passage, not absorption, supplementation of BCVFA in vivo would likely further increase BCVFA supply to the ruminant. The post-ruminal effects of BCVFA require further research.

The effects of BCVFA supplementation on bacterial AA metabolism were also investigated in Chapter 6. With BCVFA supplementation, the total AA flow leaving the vessels increased, the percentage of BCAA in bacterial AA increased, and the flow of bacterial BCAA increased. The greater total AA flow with BCVFA would be expected to increase milk production or production efficiency in dairy cattle. Higher BCAA in the bacterial AA profile could indicate higher intracellular concentration of BCAA. Branched-chain AA are important for regulation of bacteria's response to nutrient starvation, which suggests BCVFA could influence bacterial AA metabolism. Strategies to improve efficiency of bacterial AA synthesis are limited and the potential role of BCVFA supplementation on bacterial AA metabolism requires further research.

References

Aguilar, P. S., J. E. Cronan, Jr., and D. de Mendoza. 1998. A Bacillus subtilis gene induced by cold shock encodes a membrane phospholipid desaturase. J. Bacteriol. 180:2194-2200.

Allen, M. S. 2020. Review: Control of feed intake by hepatic oxidation in ruminant animals: integration of homeostasis and homeorhesis. Animal 14:s55-s64.

Allison, M. J. 1978. Production of branched-chain volatile fatty acids by certain anaerobic bacteria. Appl. Environ. Microbiol. 35:872-877.

Allison, M. J., A. L. Baetz, and J. Wiegel. 1984. Alternative pathways for biosynthesis of leucine and other amino acids in Bacteroides ruminicola and Bacteroides fragilis. Appl. Environ. Microbiol. 48:1111-1117.

Allison, M. J. and M. P. Bryant. 1963. Biosynthesis of branched-chain amino acids from branched-chain fatty acids by rumen bacteria. Arch. Biochem. Biophys. 101:269-277.

Allison, M. J., M. P. Bryant, and R. N. Doetsch. 1962a. Metabolic function of branched-chain volatile fatty acids, growth factors for Ruminococci I; incorporation of isovalerate into leucine. J. Bacteriol. 83:523-532.

Allison, M. J., M. P. Bryant, I. Katz, and M. Keeney. 1962b. Metabolic function of branched-chain volatile fatty acids, growth factors for Ruminococci. II. Biosynthesis of higher branched-chain fatty acids and aldehydes. J. Bacteriol. 83:1084-1093.

Allison, M. J., J. A. Bucklin, and I. M. Robinson. 1966. Importance of the isovalerate carboxylation pathway of leucine biosynthesis in the rumen. Appl. Microbiol. 14:807-814.

Alves, S. P., J. Santos-Silva, A. R. Cabrita, A. J. Fonseca, and R. J. Bessa. 2013. Detailed dimethylacetal and fatty acid composition of rumen content from lambs fed lucerne or concentrate supplemented with soybean oil. PLoS One 8:e58386.

Amorim Franco, T. M. and J. S. Blanchard. 2017. Bacterial Branched-Chain Amino Acid Biosynthesis: Structures, Mechanisms, and Drugability. Biochemistry 56:5849-5865.

Amorim Franco, T. M., L. Favrot, O. Vergnolle, and J. S. Blanchard. 2017. Mechanism-Based Inhibition of the Mycobacterium tuberculosis Branched-Chain Aminotransferase by d- and l-Cycloserine. ACS Chem Biol 12:1235-1244.

Andries, J. I., F. X. Buysse, D. L. De Brabander, and B. G. Cottyn. 1987. Isoacids in ruminant nutrition: Their role in ruminal and intermediary metabolism and possible influences on performances — A review. Anim. Feed Sci. Technol. 18:169-180.

Annous, B. A., L. A. Becker, D. O. Bayles, D. P. Labeda, and B. J. Wilkinson. 1997. Critical role of anteiso-C15:0 fatty acid in the growth of Listeria monocytogenes at low temperatures. App. Environ. Microbiol. 63:3887-3894.

AOAC. 1997. Official Methods of Analysis. 16th ed. Assoc. Off. Anal. Chem., Washington, DC.

AOAC. 2000. Official methods of analysis. 17th ed. Assoc. Off. Anal. Chem., Gaithersburg, MD.

Apajalahti, J., K. Vienola, K. Raatikainen, V. Holder, and C. A. Moran. 2019. Conversion of Branched-Chain Amino Acids to Corresponding Isoacids - An in vitro Tool for Estimating Ruminal Protein Degradability. Front Vet Sci 6:311.

Appuhamy, J. A., J. R. Knapp, O. Becvar, J. Escobar, and M. D. Hanigan. 2011. Effects of jugular-infused lysine, methionine, and branched-chain amino acids on milk protein synthesis in high-producing dairy cows. J. Dairy Sci. 94:1952-1960.

Appuhamy, J. A., N. A. Knoebel, W. A. Nayananjalie, J. Escobar, and M. D. Hanigan. 2012. Isoleucine and leucine independently regulate mTOR signaling and protein synthesis in MAC-T cells and bovine mammary tissue slices. J. Nutr. 142:484-491.

Arambel, M., E. Bartley, G. Dufva, T. Nagaraja, and A. Dayton. 1982. Effect of diet on amino and nucleic acids of rumen bacteria and protozoa. J. Dairy Sci. 65:2095-2101.

Atasoglu, C., A. Y. Guliye, and R. J. Wallace. 2004. Use of stable isotopes to measure de novo synthesis and turnover of amino acid-C and -N in mixed micro-organisms from the sheep rumen in vitro. Br. J. Nutr. 91:253-262.

Bach, A., Calsamiglia, S., Stern, M.D. 2005. Nitrogen metabolism in the rumen. J. Dairy Sci. 88:E9 - E21.

Bainbridge, M. L., L. K. Saldinger, J. W. Barlow, J. P. Alvez, J. Roman, and J. Kraft. 2018. Alteration of Rumen Bacteria and Protozoa Through Grazing Regime as a Tool to Enhance the Bioactive Fatty Acid Content of Bovine Milk. Front Microbiol 9:904.

Baldin, M., G. I. Zanton, and K. J. Harvatine. 2018. Effect of 2-hydroxy-4-(methylthio)butanoate (HMTBa) on risk of biohydrogenation-induced milk fat depression. J. Dairy Sci. 101:376-385.

Baldwin, R. and R. Emery. 1960. The oxidation-reduction potential of rumen contents. J. Dairy Sci. 43:506-511.

Ball, G. F. 2006. Vitamins in foods: analysis, bioavailability, and stability. CRC Press.

Banks, A. and T. P. Hilditch. 1931. The glyceride structure of beef tallows. Biochem J 25:1168-1182.

Bannink, A., J. Kogut, J. Dijkstra, J. France, E. Kebreab, A. M. Van Vuuren, and S. Tamminga. 2006. Estimation of the stoichiometry of volatile fatty acid production in the rumen of lactating cows. J. Theor. Biol. 238:36-51.

Bauchart, D., F. Legay-Carmier, M. Doreau, and B. Gaillard. 1990. Lipid metabolism of liquid-associated and solid-adherent bacteria in rumen contents of dairy cows offered lipid-supplemented diets. Br. J. Nutr. 63:563-578.

Bernard, L., C. Leroux, and Y. Chilliard. 2008. Expression and nutritional regulation of lipogenic genes in the ruminant lactating mammary gland. Adv Exp Med Biol 606:67-108.

Bionaz, M., E. Vargas-Bello-Perez, and S. Busato. 2020. Advances in fatty acids nutrition in dairy cows: from gut to cells and effects on performance. J. Anim. Sci. Biotechnol. 11:110.

Blair, B. G. and K. L. Anderson. 1999. Cellulose-inducible ultrastructural protuberances and cellulose-affinity proteins of Eubacterium cellulosolvens. Anaerobe 5:547-554.

Body, D. R. and T. Bauchop. 1985. Lipid composition of an obligately anaerobic fungus Neocallimastix frontalis isolated from a bovine rumen. Canadian journal of microbiology 31:463-466.

Brameld, J. M., J. L. Atkinson, J. C. Saunders, J. M. Pell, P. J. Buttery, and R. S. Gilmour. 1996. Effects of growth hormone administration and dietary protein intake on insulin-like growth factor I and growth hormone receptor mRNA Expression in porcine liver, skeletal muscle, and adipose tissue. J. Anim. Sci. 74:1832-1841.

Breier, B. H. 1999. Regulation of protein and energy metabolism by the somatotropic axis. Domest Anim Endocrinol 17:209-218.

Burnet, M. C., A. C. Dohnalkova, A. P. Neumann, M. S. Lipton, R. D. Smith, G. Suen, and S. J. Callister. 2015. Evaluating models of cellulose degradation by *Fibrobacter succinogenes* S85. PLoS One 10:e0143809.

Cai, Q., H. Huang, D. Qian, K. Chen, J. Luo, Y. Tian, T. Lin, and T. Lin. 2013. 13-methyltetradecanoic acid exhibits anti-tumor activity on T-cell lymphomas in vitro and in vivo by down-regulating p-AKT and activating caspase-3. PLoS One 8:e65308.

Calvo, J. M. and R. G. Matthews. 1994. The leucine-responsive regulatory protein, a global regulator of metabolism in Escherichia coli. Microbiol. Rev. 58:466-490.

Choi, K. H., R. J. Heath, and C. O. Rock. 2000. β-ketoacyl-acyl carrier protein synthase III (FabH) is a determining factor in branched-chain fatty acid biosynthesis. J. Bacteriol. 182:365-370.

Copelin, J. E., J. L. Firkins, M. T. Socha, and C. Lee. 2021. Effects of diet fermentability and supplementation of 2-hydroxy-4-(methylthio)-butanoic acid and isoacids on milk fat depression: 1. Production, milk fatty acid profile, and nutrient digestibility. J. Dairy Sci. 104:1591-1603.

Costerton, J. W. 1970. The structure and function of the cell envelope of gram-negative bacteria. Rev Can Biol 29:299-316.

Costerton, J. W., J. M. Ingram, and K. J. Cheng. 1974. Structure and function of the cell envelope of gram-negative bacteria. Bacteriol. Rev. 38:87-110.

Costerton, J. W., R. T. Irvin, and K. J. Cheng. 1981. The bacterial glycocalyx in nature and disease. Annu Rev Microbiol 35:299-324.

Croom, W. J., Jr., D. E. Bauman, and C. L. Davis. 1981. Methylmalonic acid in low-fat milk syndrome. J. Dairy Sci. 64:649-654.

Cummins, K. A. and A. H. Papas. 1985. Effect of Isocarbon-4 and Isocarbon-5 Volatile Fatty Acids on Microbial Protein Synthesis and Dry Matter Digestibility In Vitro. J. Dairy Sci. 68:2588-2595.

Cunningham, K. D., M. J. Cecava, and T. R. Johnson. 1994. Flows of Nitrogen and Amino Acids in Dairy Cows Fed Diets Containing Supplemental Feather Meal and Blood Meal. J. Dairy Sci. 77:3666-3675.

Dawson, R., N. Hemington, and G. Hazlewood. 1977. On the role of higher plant and microbial lipases in the ruminal hydrolysis of grass lipids. British Journal of Nutrition 38:225-232.

Dawson, R. M. and N. Hemington. 1974. Digestion of grass lipids and pigments in the sheep rumen. Br. J. Nutr. 32:327-340.

de Souza, J., C. M. Prom, and A. L. Lock. 2021. Altering the ratio of dietary palmitic and oleic acids affects nutrient digestibility, metabolism, and energy balance during the immediate postpartum in dairy cows. J. Dairy Sci. 104:2910-2923.

De Visser, H. and S. Tamminga. 1987. Influence of wet vs. dry by-product ingredients and addition of branched-chain volatile fatty acids and valerate to dairy diets. I. Feed intake, milk production and milk composition. NJAS wageningen journal of life sciences 35:163-175.

Dehority, B. 1966. Characterization of several bovine rumen bacteria isolated with a xylan medium. J. Bacteriol. 91:1724-1729.

Dehority, B., H. Scott, and P. Kowaluk. 1967. Volatile fatty acid requirements of cellulolytic rumen bacteria. J. Bacteriol. 94:537-543.

Dehority, B. A., R. R. Johnson, O. G. Bentley, and A. L. Moxon. 1958. Studies on the metabolism of valine, proline, leucine and isoleucine by rumen microorganisms in vitro. Arch. Biochem. Biophys. 78:15-27.

Devillard, E., F. M. McIntosh, C. J. Newbold, and R. J. Wallace. 2006. Rumen ciliate protozoa contain high concentrations of conjugated linoleic acids and vaccenic acid, yet do not hydrogenate linoleic acid or desaturate stearic acid. Br. J. Nutr. 96:697-704.

Dewhurst, R. J., J. M. Moorby, B. Vlaeminck, and V. Fievez. 2007. Apparent recovery of duodenal odd- and branched-chain fatty acids in milk of dairy cows. J. Dairy Sci. 90:1775-1780.

Doelman, J., J. J. Kim, M. Carson, J. A. Metcalf, and J. P. Cant. 2015. Branched-chain amino acid and lysine deficiencies exert different effects on mammary translational regulation. J. Dairy Sci. 98:7846-7855.

Duckett, S., J. Andrae, and F. Owens. 2002. Effect of high-oil corn or added corn oil on ruminal biohydrogenation of fatty acids and conjugated linoleic acid formation in beef steers fed finishing diets. J. Anim. Sci. 80:3353-3360.

Eibler, D., H. Abdurahman, T. Ruoff, S. Kaffarnik, H. Steingass, and W. Vetter. 2017. Unexpected Formation of Low Amounts of (R)-Configurated anteiso-Fatty Acids in Rumen Fluid Experiments. PLoS One 12:e0170788.

El-Shazly, K. 1952a. Degradation of protein in the rumen of the sheep. 1. Some volatile fatty acids, including branched-chain isomers, found in vivo. Biochemical Journal 51:640.

El-Shazly, K. 1952b. Degradation of protein in the rumen of the sheep. 2. The action of rumen micro-organisms on amino-acids. Biochemical Journal 51:647.

Enjalbert, F., S. Combes, A. Zened, and A. Meynadier. 2017. Rumen microbiota and dietary fat: a mutual shaping. J Appl Microbiol 123:782-797.

Faruque, A. J., B. D. Jarvis, and J. C. Hawke. 1974. Studies on rumen metabolism. IX. Contribution of plant lipases to the release of free fatty acids in the rumen. J Sci Food Agric 25:1313-1328.

Faulkner, M. J., B. A. Wenner, L. M. Solden, and W. P. Weiss. 2017. Source of supplemental dietary copper, zinc, and manganese affects fecal microbial relative abundance in lactating dairy cows. J. Dairy Sci. 100:1037-1044.

Felix, A., R. Cook, and J. Huber. 1980a. Isoacids and urea as a protein supplement for lactating cows fed corn silage. J. Dairy Sci. 63:1098-1103.

Felix, A., R. M. Cook, and J. T. Huber. 1980b. Effect of feeding isoacids with urea on growth and nutrient utilization by lactating cows. J. Dairy Sci. 63:1943-1946.

Firkins, J. L. 2010. Reconsidering rumen microbial consortia to enhance feed efficiency and reduce environmental impact of ruminant livestock production systems. Rev. Bras. de Zootec. 39:445-457.

Firkins, J. L. 2021. Invited Review: Advances in rumen efficiency. Appl. Anim. Sci. 37:388-403.

Firkins, J. L., C. M. Fowler, E. Devillard, and B. J. Bequette. 2015. Kinetics of microbial methionine metabolism in continuous cultures administered different methionine sources. J. Dairy Sci. 98:1178-1194.

Fox, D. G., L. Tedeschi, T. Tylutki, J. Russell, M. Van Amburgh, L. Chase, A. Pell, and T. Overton. 2004. The Cornell Net Carbohydrate and Protein System model for evaluating herd nutrition and nutrient excretion. Anim. Feed Sci. Technol. 112:29-78.

French, E. A., S. J. Bertics, and L. E. Armentano. 2012. Rumen and milk odd- and branched-chain fatty acid proportions are minimally influenced by ruminal volatile fatty acid infusions. J. Dairy Sci. 95:2015-2026.

Fuentes, M. C., S. Calsamiglia, P. W. Cardozo, and B. Vlaeminck. 2009. Effect of pH and level of concentrate in the diet on the production of biohydrogenation intermediates in a dual-flow continuous culture. J. Dairy Sci. 92:4456-4466.

Fulco, A. J. 1983. Fatty acid metabolism in bacteria. Prog Lipid Res 22:133-160.

Garton, G. A. 1959. Lipids in relation to rumen function. Proc Nutr Soc 18:112-117.

Garton, G. A., A. K. Lough, and E. Vioque. 1961. Glyceride hydrolysis and glycerol fermentation by sheep rumen contents. J Gen Microbiol 25:215-225.

Glasser, F., M. Doreau, A. Ferlay, and Y. Chilliard. 2007. Technical note: Estimation of milk fatty acid yield from milk fat data. J. Dairy Sci. 90:2302-2304.

Goering, H. K. and P. J. Van Soest. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). No. 379. US Agricultural Research Service.

Goldfine, H. 2010a. The appearance, disappearance and reappearance of plasmalogens in evolution. Prog. Lipid Res. 49:493-498.

Goldfine, H. 2010b. Membrane biogenesis. in Handbook of Hydrocarbon and Lipid Microbiology.

Goldfine, H. 2017. The anaerobic biosynthesis of plasmalogens. FEBS Lett. 591:2714-2719.

Gollop, N., B. Damri, Z. Barak, and D. M. Chipman. 1989. Kinetics and mechanism of acetohydroxy acid synthase isozyme III from Escherichia coli. Biochemistry 28:6310-6317.

Gorosito, A. R., J. B. Russell, and P. J. Van Soest. 1985. Effect of Carbon-4 and Carbon-5 Volatile Fatty Acids on Digestion of Plant Cell Wall In Vitro. J. Dairy Sci. 68:840-847.

Gram, C. 1884. Ueber die isolirte Farbung der Schizomyceten in Schnitt-und Trockenpraparaten. Fortschritte der Medicin 2:185-189.

Griinari, J. M., D. A. Dwyer, M. A. McGuire, D. E. Bauman, D. L. Palmquist, and K. V. Nurmela. 1998. Trans-octadecenoic acids and milk fat depression in lactating dairy cows. J. Dairy Sci. 81:1251-1261.

Griswold, K. E., W. H. Hoover, T. K. Miller, and W. V. Thayne. 1996. Effect of form of nitrogen on growth of ruminal microbes in continuous culture. J. Anim. Sci. 74:483-491.

Grummer, R. R. 1993. Etiology of lipid-related metabolic disorders in periparturient dairy cows. J. Dairy Sci. 76:3882-3896.

Guédon, E., P. Serror, S. D. Ehrlich, P. Renault, and C. Delorme. 2001. Pleiotropic transcriptional repressor CodY senses the intracellular pool of branched-chain amino acids in Lactococcus lactis. Mol. Microbiol. 40:1227-1239.

Gunstone, F. D. 1996. Fatty acid and lipid chemistry. Springer.

Hackmann, T. J. and J. L. Firkins. 2015a. Electron transport phosphorylation in rumen butyrivibrios: unprecedented ATP yield for glucose fermentation to butyrate. Front. Microbiol. 6:622.

Hackmann, T. J. and J. L. Firkins. 2015b. Maximizing efficiency of rumen microbial protein production. Front. Microbiol. Frontiers in Microbiology 6.

Hall, M. 2009. Analysis of starch, including maltooligosaccharides, in animal feeds: A comparison of methods and a recommended method for AOAC collaborative study. J. AOAC Int 92:42-49.

Haque, M. N., H. Rulquin, and S. Lemosquet. 2013. Milk protein responses in dairy cows to changes in postruminal supplies of arginine, isoleucine, and valine. J. Dairy Sci. 96:420-430.

Harfoot, C. G. 1978. Lipid metabolism in the rumen. Prog Lipid Res 17:21-54.

Hartinger, T., N. Gresner, and K. H. Sudekum. 2018. Does intra-ruminal nitrogen recycling waste valuable resources? A review of major players and their manipulation. J. Anim. Sci. Biotechnol. 9:33.

Hauser, H., G. P. Hazlewood, and R. M. C. Dawson. 1979. Membrane fluidity of a fatty acid auxotroph grown with palmitic acid. Nature 279:536-538.

Hazlewood, G. and R. Dawson. 1975. Isolation and properties of a phospholipid-hydrolysing bacterium from ovine rumen fluid. Microbiology 89:163-174.

Hazlewood, G. and R. M. Dawson. 1977. Acylgalactosylglycerols as a source of long-chain fatty acids for a naturally occurring rumen auxotroph. Portland Press Ltd.

Hazlewood, G. and R. M. Dawson. 1979. Characteristics of a lipolytic and fatty acid-requiring Butyrivibrio sp. isolated from the ovine rumen. Microbiology 112:15-27.

Hazlewood, G., P. Kemp, D. Lander, and R. Dawson. 1976. C 18 unsaturated fatty acid hydrogenation patterns of some rumen bacteria and their ability to hydrolyse exogenous phospholipid. British Journal of Nutrition 35:293-297.

Henderson, C. 1971. A study of the lipase produced by Anaerovibrio lipolytica, a rumen bacterium. Microbiology 65:81-89.

Hino, T. and Y. Nagatake. 1993. The effect of grass lipids on fiber digestion by mixed rumen microorganisms in vitro. Anim. Sci. Technol.(Jpn.) 64:121-128.

Hino, T. and J. B. Russell. 1985. Effect of reducing-equivalent disposal and NADH/NAD on deamination of amino acids by intact rumen microorganisms and their cell extracts. Appl. Environ. Microbiol. 50:1368-1374.

Hobson, P. N. and S. O. Mann. 1961. The isolation of glycerol-fermenting and lipolytic bacteria from the rumen of the sheep. J Gen Microbiol 25:227-240.

Hobson, P. N. and C. S. Stewart. 1988. The rumen microbial ecosystem. Springer Science & Business Media.

Horning, M. G., D. B. Martin, A. Karmen, and P. R. Vagelos. 1961. Fatty acid synthesis in adipose tissue. II. Enzymatic synthesis of branched chain and odd-numbered fatty acids. J Biol Chem 236:669-672.

Hristov, A. N., P. Huhtanen, L. M. Rode, S. N. Acharya, and T. A. McAllister. 2001. Comparison of the ruminal metabolism of nitrogen from 15N-labeled alfalfa preserved as hay or as silage. J. Dairy Sci. 84:2738-2750.

Huang, Y., J. P. Marden, C. Julien, and C. Bayourthe. 2018. Redox potential: An intrinsic parameter of the rumen environment. J. Anim. Physiol. Anim. Nutr. (Berl) 102:393-402.

Hultquist, K. M. and D. P. Casper. 2016. Effects of feeding rumen-degradable valine on milk production in late-lactating dairy cows. J. Dairy Sci. 99:1201-1215.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press, New York, London.

Hungate, R. E. and I. A. Dyer. 1956. Effect of Valeric and Isovaleric Acids on Straw Utilization by Steers. Journal of Animal Science 15:485.

Huws, S. A., E. J. Kim, A. H. Kingston-Smith, M. R. Lee, S. M. Muetzel, A. R. Cookson, C. J. Newbold, R. J. Wallace, and N. D. Scollan. 2009. Rumen protozoa are rich in polyunsaturated fatty acids due to the ingestion of chloroplasts. FEMS Microbiol Ecol 69:461-471.

Ichihara, A. and E. Koyama. 1966. Transaminase of branched chain amino acids. I. Branched chain amino acids-alpha-ketoglutarate transaminase. J Biochem 59:160-169.

Ifkovits, R. W. and H. S. Ragheb. 1968. Cellular fatty acid composition and identification of rumen bacteria. Applied microbiology 16:1406-1413.

Jackson, D. R., C. D. Cassilly, D. R. Plichta, H. Vlamakis, H. Liu, S. B. Melville, R. J. Xavier, and J. Clardy. 2021. Plasmalogen biosynthesis by anaerobic bacteria: identification of a two-gene operon responsible for plasmalogen production in *Clostridium perfringens*. ACS Chem. Biol. 16:6-13.

Jarrige, R., E. Grenet, C. Demarquilly, and J. Besle. 1995. Les constituants de l'appareil végétatif des plantes fourragères. Nutrition des ruminants domestiques: Ingestion et digestion:25.

Jenkins, T. C. 1993. Lipid metabolism in the rumen. J. Dairy Sci. 76:3851-3863.

Jenkins, T. C. 2010. Technical note: common analytical errors yielding inaccurate results during analysis of fatty acids in feed and digesta samples. J. Dairy Sci. 93:1170-1174.

Jenkins, T. C. and D. L. Palmquist. 1984. Effect of fatty acids or calcium soaps on rumen and total nutrient digestibility of dairy rations. J. Dairy Sci. 67:978-986.

Jenkins, T. C., R. J. Wallace, P. J. Moate, and E. E. Mosley. 2008. Board-invited review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. J. Anim. Sci. 86:397-412.

Johnson, T. R., M. J. Cecava, E. B. Sheiss, and K. D. Cunningham. 1994. Addition of ruminally degradable crude protein and branched-chain volatile fatty acids to diets containing hydrolyzed feather meal and blood meal for lactating cows. J. Dairy Sci. 77:3676-3682.

Kaiser, J. C. and D. E. Heinrichs. 2018. Branching out: alterations in bacterial physiology and virulence due to branched-chain amino acid deprivation. MBio 9:e01188-01118.

Kajikawa, H., M. Mitsumori, and S. Ohmomo. 2002. Stimulatory and inhibitory effects of protein amino acids on growth rate and efficiency of mixed ruminal bacteria. J. Dairy Sci. 85:2015-2022.

Kajikawa, H., M. Mitsumori, K. Tajima, and M. Kurihara. 2005. Short Communication: Amino acids antagonistic to the amino acids inhibitory for growth rate of mixed ruminal bacteria. J. Dairy Sci. 88:2601-2603.

Kaneda, T. 1967. Fatty Acids in the Genus Bacillus I. Iso- and Anteiso-Fatty Acids as Characteristic Constituents of Lipids in 10 Species. J. Bacteriol. 93:894-903.

Kaneda, T. 1977. Fatty acids of the genus Bacillus: an example of branched-chain preference. Bacteriol. Rev. 41:391-418.

Kaneda, T. 1991. Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol. Rev. 55:288-302.

Kanehisa, M., S. Goto, Y. Sato, M. Furumichi, and M. Tanabe. 2012. KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Res. 40:D109-D114.

Katz, I. and M. Keeney. 1964. The Isolation of Fatty Aldehydes from Rumen-Microbial Lipid. Biochim Biophys Acta 84:128-132.

Kazakov, A. E., D. A. Rodionov, E. Alm, A. P. Arkin, I. Dubchak, and M. S. Gelfand. 2009. Comparative genomics of regulation of fatty acid and branched-chain amino acid utilization in proteobacteria. J. Bacteriol. 191:52-64.

Kelly, W. J., R. I. Mackie, G. T. Attwood, P. H. Janssen, T. A. McAllister, and S. C. Leahy. 2022. Hydrogen and formate production and utilisation in the rumen and the human colon. Anim Microbiome 4:22.

Kemp, P. and D. J. Lander. 1984. Hydrogenation in vitro of α-linolenic acid to stearic acid by mixed cultures of pure strains of rumen bacteria. Microbiology 130:527-533.

Kemp, P., D. J. Lander, and C. G. Orpin. 1984. The lipids of the rumen fungus Piromonas communis. J Gen Microbiol 130:27-37.

Kemp, P., R. W. White, and D. J. Lander. 1975. The hydrogenation of unsaturated fatty acids by five bacterial isolates from the sheep rumen, including a new species. J Gen Microbiol 90:100-114.

Kepler, C. R., W. P. Tucker, and S. B. Tove. 1970. Biohydrogenation of unsaturated fatty acids. IV. Substrate specificity and inhibition of linoleate delta-12-cis, delta-11-trans-isomerase from Butyrivibrio fibrisolvens. J Biol Chem 245:3612-3620.

Kim, E. 2009. Mechanisms of amino acid sensing in mTOR signaling pathway. Nutr. Res. Pract. 3:64-71.

Kim, S. H., J. H. Park, K. H. Kang, J. H. Lee, C. K. Park, C. M. Cho, W. Y. Tak, Y. O. Kweon, S. K. Kim, Y. H. Choi, W. S. Yoo, and H. I. Bae. 2005. Gastric tuberculosis presenting as a submucosal tumor. Gastrointest Endosc 61:319-322.

Klusmeyer, T. H., J. H. Clark, J. L. Vicini, M. R. Murphy, and G. C. Fahey, Jr. 1987. Effects of feeding or infusing ammonium salts of volatile fatty acids on ruminal fermentation, plasma characteristics, and milk production of cows. J. Dairy Sci. 70:50-63.

Klusmeyer, T. H., G. L. Lynch, J. H. Clark, and D. R. Nelson. 1991. Effects of Calcium Salts of Fatty Acids and Proportion of Forage in Diet on Ruminal Fermentation and Nutrient Flow to Duodenum of Cows. J. Dairy Sci. 74:2220-2232.

Koga, Y. and H. Morii. 2005. Recent advances in structural research on ether lipids from archaea including comparative and physiological aspects. Biosci Biotechnol Biochem 69:2019-2034.

Kone, P., P. F. Machado, and R. M. Cook. 1989. Effect of the combination of monensin and isoacids on rumen fermentation in vitro. J. Dairy Sci. 72:2767-2771.

Kopecny, J., M. Zorec, J. Mrazek, Y. Kobayashi, and R. Marinsek-Logar. 2003. Butyrivibrio hungatei sp. nov. and Pseudobutyrivibrio xylanivorans sp. nov., butyrate-producing bacteria from the rumen. Int. J. Syst. Evol. Microbiol. 53:201-209.

Korhonen, M., A. Vanhatalo, and P. Huhtanen. 2002. Evaluation of isoleucine, leucine, and valine as a second-limiting amino acid for milk production in dairy cows fed grass silage diet. J. Dairy Sci. 85:1533-1545.

Krishtalik, L. I. 2003. pH-dependent redox potential: how to use it correctly in the activation energy analysis. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1604:13-21.

Kuiper, P. J. 1970. Lipids in alfalfa leaves in relation to cold hardiness. Plant Physiol 45:684-686.

Landry, J. and S. Delhaye. 1992. Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. J. Agric. Food Chem. 40:776-779.

Langworthy, T. A. 1985. Lipids of archaebacteria. Vol. 8. The Bacteria. Academic Press, Inc., New York.

Lapierre, H., S. Binggeli, M. Sok, D. Pellerin, and D. R. Ouellet. 2019. Estimation of correction factors to determine the true amino acid concentration of protein after a 24-hour hydrolysis. J. Dairy Sci. 102:1205-1212.

Lassiter, C., R. Emery, and C. Duncan. 1958. The effect of valeric acid and a combination of valeric and iso-valeric acids on the performance of milking cows. Journal of Animal Science 17:358-362.

Latham, M. J., B. E. Brooker, G. L. Pettipher, and P. J. Harris. 1978. Ruminococcus flavefaciens Cell Coat and Adhesion to Cotton Cellulose and to Cell Walls in Leaves of Perennial Ryegrass (Lolium perenne). Appl. Environ. Microbiol. 35:156-165.

Latham, M. J., J. E. Storry, and M. E. Sharpe. 1972. Effect of low-roughage diets on the microflora and lipid metabolism in the rumen. Applied microbiology 24:871-877.

Leal Yepes, F. A., S. Mann, T. R. Overton, E. Behling-Kelly, D. V. Nydam, and J. J. Wakshlag. 2021. Hepatic effects of rumen-protected branched-chain amino acids with or without propylene glycol supplementation in dairy cows during early lactation. J. Dairy Sci. 104:10324-10337.

Leal Yepes, F. A., S. Mann, T. R. Overton, C. M. Ryan, L. S. Bristol, G. E. Granados, D. V. Nydam, and J. J. Wakshlag. 2019. Effect of rumen-protected branched-chain amino acid supplementation on production- and energy-related metabolites during the first 35 days in milk in Holstein dairy cows. J. Dairy Sci. 102:5657-5672.

Lee, C., J. E. Copelin, T. Park, K. E. Mitchell, J. L. Firkins, M. T. Socha, and D. Luchini. 2021. Effects of diet fermentability and supplementation of 2-hydroxy-4-(methylthio)-butanoic acid and isoacids on milk fat depression: 2. Ruminal fermentation, fatty acid, and bacterial community structure. J. Dairy Sci. 104:1604-1619.

Lehner, R. and A. Kuksis. 1996. Biosynthesis of triacylglycerols. Prog Lipid Res 35:169-201.

Li, X., H. Chen, Y. Guan, X. Li, L. Lei, J. Liu, L. Yin, G. Liu, and Z. Wang. 2013. Acetic acid activates the AMP-activated protein kinase signaling pathway to regulate lipid metabolism in bovine hepatocytes. PLoS One 8:e67880.

Li, Y., G. Florova, and K. A. Reynolds. 2005. Alteration of the fatty acid profile of Streptomyces coelicolor by replacement of the initiation enzyme 3-ketoacyl acyl carrier protein synthase III (FabH). J. Bacteriol. 187:3795-3799.

Liu, C. G., C. Xue, Y. H. Lin, and F. W. Bai. 2013. Redox potential control and applications in microaerobic and anaerobic fermentations. Biotechnol. Adv. 31:257-265.

Liu, Q., C. Wang, G. Guo, W. J. Huo, S. L. Zhang, C. X. Pei, Y. L. Zhang, and H. Wang. 2018a. Effects of branched-chain volatile fatty acids on lactation performance and mRNA expression of genes related to fatty acid synthesis in mammary gland of dairy cows. Animal 12:2071-2079.

Liu, Q., C. Wang, G. Guo, W. J. Huo, Y. L. Zhang, C. X. Pei, S. L. Zhang, and H. Wang. 2018b. Effects of branched-chain volatile fatty acids supplementation on growth performance, ruminal fermentation, nutrient digestibility, hepatic lipid content and gene expression of dairy calves. Anim. Feed Sci. Technol. 237:27-34.

Liu, Q., C. Wang, G. Guo, W. J. Huo, Y. L. Zhang, C. X. Pei, S. L. Zhang, and H. Wang. 2018c. Effects of isovalerate supplements on morphology and functional gene expression of rumen mucosa in pre- and post-weaning dairy calves. Animal 12:491-500.

Liu, Q., C. Wang, Y. X. Huang, K. H. Dong, W. Z. Yang, S. L. Zhang, and H. Wang. 2009a. Effects of isovalerate on ruminal fermentation, urinary excretion of purine derivatives and digestibility in steers. J. Anim. Physiol. Anim. Nutr. (Berl) 93:716-725.

Liu, Q., C. Wang, H. Li, G. Guo, W. Huo, C. Pei, S. Zhang, and H. Wang. 2017. Effects of dietary protein levels and rumen-protected pantothenate on ruminal fermentation, microbial enzyme activity and bacteria population in Blonde d'Aquitaine× Simmental beef steers. Anim. Feed Sci. Technol. 232:31-39.

Liu, Q., C. Wang, H. Li, G. Guo, W. Huo, S. Zhang, Y. Zhang, C. Pei, and H. Wang. 2018d. Effects of dietary protein level and rumen-protected pantothenate on nutrient digestibility, nitrogen balance, blood metabolites and growth performance in beef calves. Journal of Animal and Feed Sciences 27:202-210.

Liu, Q., C. Wang, C. X. Pei, H. Y. Li, Y. X. Wang, S. L. Zhang, Y. L. Zhang, J. P. He, H. Wang, W. Z. Yang, Y. S. Bai, Z. G. Shi, and X. N. Liu. 2014. Effects of isovalerate supplementation on microbial status and rumen enzyme profile in steers fed on corn stover based diet. Livest. Sci. 161:60-68.

Liu, Q., C. Wang, W. Z. Yang, B. Zhang, X. M. Yang, D. C. He, P. Zhang, K. H. Dong, and Y. X. Huang. 2009b. Effects of isobutyrate on rumen fermentation, lactation performance and plasma characteristics in dairy cows. Anim. Feed Sci. Technol. 154:58-67.

Liu, Q., C. Wang, Y. L. Zhang, C. X. Pei, S. L. Zhang, H. Q. Li, G. Guo, Y. J. Huo, W. Z. Yang, and H. Wang. 2016. Effects of 2-methylbutyrate supplementation on growth performance and ruminal development in pre- and post-weaned dairy calves. Anim. Feed Sci. Technol. 216:129-137.

Lock, A. L. and D. E. Bauman. 2004. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. Lipids 39:1197-1206.

Mackle, T. R., D. A. Dwyer, and D. E. Bauman. 1999. Effects of branched-chain amino acids and sodium caseinate on milk protein concentration and yield from dairy cows. J. Dairy Sci. 82:161-171.

Maia, M. R., L. C. Chaudhary, C. S. Bestwick, A. J. Richardson, N. McKain, T. R. Larson, I. A. Graham, and R. J. Wallace. 2010. Toxicity of unsaturated fatty acids to the biohydrogenating ruminal bacterium, Butyrivibrio fibrisolvens. BMC Microbiol 10:52.

Maia, M. R., L. C. Chaudhary, L. Figueres, and R. J. Wallace. 2007. Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. Antonie Van Leeuwenhoek 91:303-314.

Mannelli, F., A. Cappucci, F. Pini, R. Pastorelli, F. Decorosi, L. Giovannetti, M. Mele, S. Minieri, G. Conte, M. Pauselli, S. Rapaccini, C. Viti, and A. Buccioni. 2018. Effect of different types of olive oil pomace dietary supplementation on the rumen microbial community profile in Comisana ewes. Sci. Rep. 8:8455.

Massey, L. K., J. R. Sokatch, and R. S. Conrad. 1976. Branched-chain amino acid catabolism in bacteria. Bacteriol. Rev. 40:42-54.

McDowell, G. H., J. M. Gooden, D. Leenanuruksa, M. Jois, and A. W. English. 1987. Effects of exogenous growth hormone on milk production and nutrient uptake by muscle and mammary tissues of dairy cows in mid-lactation. Aust J Biol Sci 40:295-306.

McKain, N., K. J. Shingfield, and R. J. Wallace. 2010. Metabolism of conjugated linoleic acids and 18 : 1 fatty acids by ruminal bacteria: products and mechanisms. Microbiology 156:579-588.

Minato, H., Ishibashi, S., Hamaoka, T. 1988. Cellular fatty acid and sugar composition of representative strains of rumen bacteria. J. Gen. Appl. Microbiol 34:303-319.

Miron, J., D. Ben-Ghedalia, and M. Morrison. 2001. Invited review: adhesion mechanisms of rumen cellulolytic bacteria. J. Dairy Sci. 84:1294-1309.

Miura, H., M. Horiguchi, and T. Matsumoto. 1980. Nutritional interdependence among rumen bacteria, Bacteroides amylophilus, Megasphaera elsdenii, and Ruminococcus albus. Appl. Environ. Microbiol. 40:294-300.

Miyagawa, E., Azuma, R., Suto, T. 1979. Cellular fatty acid composition in gram-negative obligately anaerobic rods. J. Gen. Appl. Microbiol. 25:41-51.

Mizrahi, I., R. J. Wallace, and S. Moraïs. 2021. The rumen microbiome: balancing food security and environmental impacts. Nature Reviews Microbiology 19:553-566.

Molkentin, J. and D. Precht. 1995. Optimized analysis oftrans-octadecenoic acids in edible fats. Chromatographia 41:267-272.

Montgomery, L., B. Flesher, and D. Stahl. 1988. Transfer of Bacteroides succinogenes (Hungate) to Fibrobacter gen. nov. as Fibrobacter succinogenes comb. nov. and description of Fibrobacter intestinalis sp. nov. International Journal of Systematic and Evolutionary Microbiology 38:430-435.

Monticello, D. J. and R. N. Costilow. 1982. Interconversion of valine and leucine by Clostridium sporogenes. J. Bacteriol. 152:946-949.

Moon, C. D., D. M. Pacheco, W. J. Kelly, S. C. Leahy, D. Li, J. Kopečný, and G. T. Attwood. 2008. Reclassification of Clostridium proteoclasticum as Butyrivibrio proteoclasticus comb. nov., a butyrate-producing ruminal bacterium. International journal of systematic and evolutionary microbiology 58:2041-2045.

Moon, M. and K. L. Anderson. 2001. Eubacterium cellulosolvens Alters its Membrane Protein, Lipoprotein, and Fatty Acid Composition in Response to Growth on Cellulose. Anaerobe 7:227-236.

Morais, S. and I. Mizrahi. 2019. Islands in the stream: from individual to communal fiber degradation in the rumen ecosystem. FEMS Microbiol. Rev. 43:362-379.

Myers, J. W. 1961. Dihydroxy acid dehydrase: an enzyme involved in the biosynthesis of isoleucine and valine. J Biol Chem 236:1414-1418.

Neumann-Schaal, M., D. Jahn, and K. Schmidt-Hohagen. 2019. Metabolism the Difficile Way: The Key to the Success of the Pathogen Clostridioides difficile. Front Microbiol 10:219.

Noble, R. C., J. H. Moore, and C. G. Harfoot. 1974. Observations on the pattern on biohydrogenation of esterified and unesterified linoleic acid in the rumen. Br. J. Nutr. 31:99-108.

NRC. 2001. Nutrient requirements of dairy cattle. National Academy of Sciences, Washington (DC).

Oberg, T. S., R. E. Ward, J. L. Steele, and J. R. Broadbent. 2012. Identification of plasmalogens in the cytoplasmic membrane of Bifidobacterium animalis subsp. lactis. Appl. Environ. Microbiol. 78:880-884.

Omphalius, C., S. Lemosquet, D. R. Ouellet, L. Bahloul, and H. Lapierre. 2020. Postruminal infusions of amino acids or glucose affect metabolisms of splanchnic, mammary, and other peripheral tissues and drive amino acid use in dairy cows. J. Dairy Sci. 103:2233-2254.

Or-Rashid, M. M., N. E. Odongo, and B. W. McBride. 2007. Fatty acid composition of ruminal bacteria and protozoa, with emphasis on conjugated linoleic acid, vaccenic acid, and odd-chain and branched-chain fatty acids. J. Anim. Sci. 85:1228-1234.

Otterby, D. E., D. G. Johnson, R. Towns, R. M. Cook, R. A. Erdman, H. H. Van Horn, J. A. Rogers, and W. A. Clark. 1990. Dose response of dairy cows to ammonium salts of volatile fatty acids. J. Dairy Sci. 73:2168-2178.

Papas, A. M., S. R. Ames, R. M. Cook, C. J. Sniffen, C. E. Polan, and L. Chase. 1984. Production responses of dairy cows fed diets supplemented with ammonium salts of iso C-4 and C-5 acids. J. Dairy Sci. 67:276-293.

Parsons, J. B. and C. O. Rock. 2013. Bacterial lipids: metabolism and membrane homeostasis. Prog. Lipid Res. 52:249-276.

Peirce-Sandner, S. B., A. M. Papas, J. A. Rogers, T. F. Sweeney, K. A. Cummins, H. R. Conrad, and L. D. Muller. 1985. Supplementation of dairy cow diets with ammonium salts of volatile fatty acids. J. Dairy Sci. 68:2895-2907.

Piao, H., M. Lachman, S. Malfatti, A. Sczyrba, B. Knierim, M. Auer, S. G. Tringe, R. I. Mackie, C. J. Yeoman, and M. Hess. 2014. Temporal dynamics of fibrolytic and methanogenic rumen microorganisms during in situ incubation of switchgrass determined by 16S rRNA gene profiling. Frontiers in Microbiology 5:307.

Polan, C. E., J. J. McNeill, and S. B. Tove. 1964. Biohydrogenation of Unsaturated Fatty Acids by Rumen Bacteria. J. Bacteriol. 88:1056-1064.

Prado, L. A., P. Schmidely, P. Noziere, and A. Ferlay. 2019. Milk saturated fatty acids, odd- and branched-chain fatty acids, and isomers of C18:1, C18:2, and C18:3n-3 according to their duodenal flows in dairy cows: A meta-analysis approach. J. Dairy Sci. 102:3053-3070.

Qiu, X., A. E. Choudhry, C. A. Janson, M. Grooms, R. A. Daines, J. T. Lonsdale, and S. S. Khandekar. 2005. Crystal structure and substrate specificity of the beta-ketoacyl-acyl carrier protein synthase III (FabH) from Staphylococcus aureus. Protein Sci 14:2087-2094.

Radhakrishnan, A. N., R. P. Wagner, and E. E. Snell. 1960. Biosynthesis of Valine and Isoleucine: III. α-KETO-β-HYDROXY ACID REDUCTASE AND α-HYDROXY-β-KETO ACID REDUCTOISOMERASE. Journal of Biological Chemistry 235:2322-2331.

Ramirez Ramirez, H. A., K. J. Harvatine, and P. J. Kononoff. 2016. Short communication: Forage particle size and fat intake affect rumen passage, the fatty acid profile of milk, and milk fat production in dairy cows consuming dried distillers grains with solubles. J. Dairy Sci. 99:392-398.

Ran-Ressler, R. R., D. Sim, A. M. O'Donnell-Megaro, D. E. Bauman, D. M. Barbano, and J. T. Brenna. 2011. Branched chain fatty acid content of United States retail cow's milk and implications for dietary intake. Lipids 46:569-576.

Ratnayake-Lecamwasam, M., P. Serror, K.-W. Wong, and A. L. Sonenshein. 2001. Bacillus subtilis CodY represses early-stationary-phase genes by sensing GTP levels. Genes & Dev. 15:1093-1103.

Rezanka, T., Z. Kresinova, I. Kolouchova, and K. Sigler. 2012. Lipidomic analysis of bacterial plasmalogens. Folia Microbiol (Praha) 57:463-472.

Rico, D. E. and K. J. Harvatine. 2013. Induction of and recovery from milk fat depression occurs progressively in dairy cows switched between diets that differ in fiber and oil concentration. J. Dairy Sci. 96:6621-6630.

Robinson, I. M. and M. J. Allison. 1969. Isoleucine biosynthesis from 2-methylbutyric acid by anaerobic bacteria from the rumen. J. Bacteriol. 97:1220-1226.

Robinson, P., S. Tamminga, and A. Van Vuuren. 1987a. Influence of wet vs. dry by-product ingredients and addition of branched-chain volatile fatty acids and valerate to dairy diets. 2. Rumen fermentation and milk production. NJAS wageningen journal of life sciences 35:447-458.

Robinson, P. H., S. Tamminga, and A. M. v. Vuuren. 1987b. Influence of wet vs. dry by-product ingredients and addition of branched-chain volatile fatty acids and valerate to dairy diets. 3. Kinetics of rumen ingesta turnover, whole tract digestibility, and some plasma hormone concentrations. Netherlands Journal of Agricultural Science 35:459-471.

Rodriguez, J., R. Kleerebezem, J. M. Lema, and M. C. van Loosdrecht. 2006. Modeling product formation in anaerobic mixed culture fermentations. Biotechnol. Bioeng. 93:592-606.

Rogers, J., W. Clark, D. Ferraro, A. Spearow, V. Blumer, C. Dougherty Jr, R. Cook, A. Jimenez, D. Dildey, and L. Armentano. 1989. Milk production of dairy cows fed ammonium and calcium salts of volatile fatty acids on 43 commercial dairy farms. J. Dairy Sci. 72:270-283.

Rogosa, M. 1971. Transfer of Peptostreptococcus elsdenii Gutierrez et al. to a new genus, Megasphaera [M. elsdenii (Gutierrez et al.) comb. nov.]. Int. J. Syst. Evol. Microbiol. 21:187-189.

Roman-Garcia, Y., B. L. Denton, K. E. Mitchell, C. Lee, M. T. Socha, and J. L. Firkins. 2021a. Conditions stimulating neutral detergent fiber degradation by dosing branched-chain volatile fatty acids. I: Comparison with branched-chain amino acids and forage source in ruminal batch cultures. J. Dairy Sci. 104:6739-6755.

Roman-Garcia, Y., K. E. Mitchell, B. L. Denton, C. Lee, M. T. Socha, B. A. Wenner, and J. L. Firkins. 2021b. 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. J. Dairy Sci. 104:9853-9867.

Roman-Garcia, Y., K. E. Mitchell, C. Lee, M. T. Socha, T. Park, B. A. Wenner, and J. L. Firkins. 2021c. 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. J. Dairy Sci. 104:9868-9885.

Rosener, D. L. and E. K. Uhlenhopp. 1987. Isoacids-An Overview. Iowa State University Veterinarian 49:4.

Roughan, P. G. and R. D. Batt. 1969. The glycerolipid composition of leaves. Phytochemistry 8:363-369.

Rudman, D. and A. Meister. 1953. Transamination in Escherichia coli. Journal of Biological Chemistry 200:591-604.

Rulquin, H. and P. M. Pisulewski. 2006. Effects of graded levels of duodenal infusions of leucine on mammary uptake and output in lactating dairy cows. J. Dairy Res. 73:328-339.

Russell, J. B. and C. J. Sniffen. 1984. Effect of carbon-4 and carbon-5 volatile fatty acids on growth of mixed rumen bacteria in vitro. J. Dairy Sci. 67:987-994.

Saluzzi, L., A. Smith, and C. S. Stewart. 1993. Analysis of bacterial phospholipid markers and plant monosaccharides during forage degradation by Ruminococcus flavefaciens and Fibrobacter succinogenes in co-culture. J Gen Microbiol 139:2865-2873.

Seshadri, R., S. C. Leahy, G. T. Attwood, K. H. Teh, S. C. Lambie, A. L. Cookson, E. A. Eloe-Fadrosh, G. A. Pavlopoulos, M. Hadjithomas, and N. J. Varghese. 2018. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. Nat. Biotechnol. 36:359-367.

Shah, H. N. and D. M. Collins. 1990. Prevotella, a new genus to include Bacteroides melaninogenicus and related species formerly classified in the genus Bacteroides. International Journal of Systematic and Evolutionary Microbiology 40:205-208.

Sheridan, P. O., P. Louis, E. Tsompanidou, S. Shaw, H. J. Harmsen, S. H. Duncan, H. J. Flint, and A. W. Walker. 2022. Distribution, organization and expression of genes concerned with anaerobic lactate utilization in human intestinal bacteria. Microbial genomics 8.

Shorland, F. 1963. The distribution of fatty acids in plant lipids. Pages 253-311 in Chemical plant taxonomy. Elsevier.

Sinensky, M. 1971. Temperature control of phospholipid biosynthesis in Escherichia coli. J. Bacteriol. 106:449-455.

Sinensky, M. 1974. Homeoviscous adaptation--a homeostatic process that regulates the viscosity of membrane lipids in Escherichia coli. Proc Natl Acad Sci U S A 71:522-525.

Singh, A. K., Y. M. Zhang, K. Zhu, C. Subramanian, Z. Li, R. K. Jayaswal, C. Gatto, C. O. Rock, and B. J. Wilkinson. 2009. FabH selectivity for anteiso branched-chain fatty acid precursors in low-temperature adaptation in Listeria monocytogenes. FEMS Microbiol Lett 301:188-192.

Smith, S. 1994. The animal fatty acid synthase: one gene, one polypeptide, seven enzymes. FASEB J 8:1248-1259.

Stackebrandt, E. and H. Hippe. 1986. Transfer of *Bacteroides amylophilus* to a new genus *Ruminobacter* gen. nov., nom. rev. as *Ruminobacter amylophilus* comb. nov. Syst. Appl. Microbiol. 8:204-207.

Stewart, C., H. Flint, and M. Bryant. 1997. The rumen bacteria. Pages 10-72 in The rumen microbial ecosystem. Springer.

Stieglitz, B. I. and J. M. Calvo. 1974. Distribution of the isopropylmalate pathway to leucine among diverse bacteria. J. Bacteriol. 118:935-941.

Styring, A. K., A. Kuhl, T. D. Knowles, R. A. Fraser, A. Bogaard, and R. P. Evershed. 2012. Practical considerations in the determination of compound-specific amino acid delta15N values in animal and plant tissues by gas chromatography-combustion-isotope ratio mass spectrometry, following derivatisation to their N-acetylisopropyl esters. Rapid Commun. Mass Spectrom. 26:2328-2334.

Sukhija, P. S. and D. Palmquist. 1988. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J. Agric. Food Chem. 36:1202-1206.

Terry, S. A., A. Badhan, Y. Wang, A. V. Chaves, and T. A. McAllister. 2019. Fibre digestion by rumen microbiota—a review of recent metagenomic and metatranscriptomic studies. Canadian Journal of Animal Science 99:678-692.

Tice, E., M. Eastridge, and J. Firkins. 1994. Raw soybeans and roasted soybeans of different particle sizes. 2. Fatty acid utilization by lactating cows. J. Dairy Sci. 77:166-180.

Trautmann, A., L. Schleicher, S. Deusch, J. Gätgens, J. Steuber, and J. Seifert. 2020. Short-chain fatty acids modulate metabolic pathways and membrane lipids in Prevotella bryantii B14. Proteomes 8:28.

Tremolieres, A. 1970. Lipids of photosynthetic tissues. Annee Biol 9:113-156.

Tsubota, H. and S. Hoshino. 1969. Transaminase activity in sheep rumen content. J. Dairy Sci. 52:2024-2028.

Umbarger, H. E. 1956. Evidence for a negative-feedback mechanism in the biosynthesis of isoleucine. Science 123:848.

Umbarger, H. E. and B. Brown. 1958. Isoleucine and valine metabolism in Escherichia coli. VII. A negative feedback mechanism controlling isoleucine biosynthesis. J Biol Chem 233:415-420.

Umbarger, H. E., B. Brown, and E. J. Eyring. 1960. Isoleucine and valine metabolism in Escherichia coli. IX. Utilization of acetolactate and acetohydroxybutyrate. J Biol Chem 235:1425-1432.

Urrutia, N. L. and K. J. Harvatine. 2017. Acetate dose-dependently stimulates milk fat synthesis in lactating dairy cows. J. Nutr. 147:763-769.

Van Soest, P., J. Robertson, and B. Lewis. 1991a. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991b. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.

Ventto, L., H. Leskinen, P. Kairenius, T. Stefanski, A. R. Bayat, J. Vilkki, and K. J. Shingfield. 2017. Diet-induced milk fat depression is associated with alterations in ruminal biohydrogenation pathways and formation of novel fatty acid intermediates in lactating cows. Br. J. Nutr. 117:364-376.

Verbeke, R., M. Lauryssens, G. Peeters, and A. T. James. 1959. Incorporation of DL-[1-14C]leucine and [1-14C]liso valeric acid into milk constituents by the perfused cow's udder. Biochem J 73:24-29.

Vinogradov, E., E. E. Egbosimba, M. B. Perry, J. S. Lam, and C. W. Forsberg. 2001. Structural analysis of the carbohydrate components of the outer membrane of the

lipopolysaccharide-lacking cellulolytic ruminal bacterium Fibrobacter succinogenes S85. European journal of biochemistry 268:3566-3576.

Viviani, R. 1970. Metabolism of long-chain fatty acids in the rumen. Adv Lipid Res 8:267-346.

Vlaeminck, B., V. Fievez, A. R. J. Cabrita, A. J. M. Fonseca, and R. J. Dewhurst. 2006. Factors affecting odd- and branched-chain fatty acids in milk: A review. Anim. Feed Sci. Technol. 131:389-417.

Vlaeminck, B., R. Gervais, M. M. Rahman, F. Gadeyne, M. Gorniak, M. Doreau, and V. Fievez. 2015. Postruminal synthesis modifies the odd- and branched-chain fatty acid profile from the duodenum to milk. J. Dairy Sci. 98:4829-4840.

Völker, D., L. Hüther, G. Daş, and H. Abel. 2011. Pantothenic acid supplementation to support rumen microbes? Archives of animal nutrition 65:163-173.

Walker, N., C. Newbold, and R. Wallace. 2005. Nitrogen metabolism in the rumen. Pages 71-115 in Nitrogen and phosphorus nutrition of cattle. CABI Publishing, Cambridge, MA.

Wallace, R. J., L. C. Chaudhary, N. McKain, N. R. McEwan, A. J. Richardson, P. E. Vercoe, N. D. Walker, and D. Paillard. 2006. Clostridium proteoclasticum: a ruminal bacterium that forms stearic acid from linoleic acid. FEMS Microbiology Letters 265:195-201.

Wang, C., Q. Liu, G. Guo, W. J. Huo, C. X. Pei, S. L. Zhang, and H. Wang. 2018. Effects of concentrate-to-forage ratios and 2-methylbutyrate supplementation on ruminal fermentation, bacteria abundance and urinary excretion of purine derivatives in Chinese Simmental steers. J. Anim. Physiol. Anim. Nutr. (Berl) 102:901-909.

Wang, C., Q. Liu, G. Guo, W. J. Huo, Y. L. Zhang, C. X. Pei, and S. L. Zhang. 2019. Effects of rumen-protected folic acid and branched-chain volatile fatty acids supplementation on lactation performance, ruminal fermentation, nutrient digestion and blood metabolites in dairy cows. Anim. Feed Sci. Technol. 247:157-165.

Wang, C., Q. Liu, C. X. Pei, H. Y. Li, Y. X. Wang, H. Wang, Y. S. Bai, Z. G. Shi, X. N. Liu, and P. Li. 2012. Effects of 2-methylbutyrate on rumen fermentation, ruminal enzyme activities, urinary excretion of purine derivatives and feed digestibility in steers. Livest. Sci. 145:160-166.

Wang, C., Q. Liu, Y. L. Zhang, C. X. Pei, S. L. Zhang, Y. X. Wang, W. Z. Yang, Y. S. Bai, Z. G. Shi, and X. N. Liu. 2015. Effects of isobutyrate supplementation on ruminal microflora, rumen enzyme activities and methane emissions in Simmental steers. J. Anim. Physiol. Anim. Nutr. 99:123-131.

Wang Meng-zhi, W. H.-r., Cao Heng-chun, Li Guo-xiang and Zhang Jie. 2008. Effects of Limiting Amino Acids on Rumen Fermentation and Microbial Community In vitro. Agricultural Sciences in China 7:1524-1531.

Ward, P. F., T. W. Scott, and R. M. Dawson. 1964. The hydrogenation of unsaturated fatty acids in the ovine digestive tract. Biochem J 92:60-68.

Wegner, G. and E. Foster. 1963. Incorporation of isobutyrate and valerate into cellular plasmalogen by Bacteroides succinogenes. Journal of bacteriology 85:53-61.

Weimer, P. J., N. P. Price, O. Kroukamp, L. M. Joubert, G. M. Wolfaardt, and W. H. Van Zyl. 2006. Studies of the extracellular glycocalyx of the anaerobic cellulolytic bacterium Ruminococcus albus 7. Appl. Environ. Microbiol. 72:7559-7566.

Weld, K. A. and L. E. Armentano. 2017. The effects of adding fat to diets of lactating dairy cows on total-tract neutral detergent fiber digestibility: A meta-analysis. J. Dairy Sci. 100:1766-1779.

Weller, R. A. and A. F. Pilgrim. 1974. Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. Br. J. Nutr. 32:341-351.

Wenner, B. A., J. de Souza, F. Batistel, T. J. Hackmann, Z. Yu, and J. L. Firkins. 2017. Association of aqueous hydrogen concentration with methane production in continuous cultures modulated to vary pH and solids passage rate. J. Dairy Sci. 100:5378-5389.

Wenner, B. A., B. K. Wagner, N. R. St-Pierre, Z. T. Yu, and J. L. Firkins. 2020. Inhibition of methanogenesis by nitrate, with or without defaunation, in continuous culture. J. Dairy Sci. 103:7124-7140.

White, R. R., Y. Roman-Garcia, and J. L. Firkins. 2016. Meta-analysis of postruminal microbial nitrogen flows in dairy cattle. II. Approaches to and implications of more mechanistic prediction. J. Dairy Sci. 99:7932-7944.

White, R. W., P. Kemp, and R. M. Dawson. 1970. Isolation of a rumen bacterium that hydrogenates oleic acid as well as linoleic acid and linolenic acid. Biochem J 116:767-768.

Whitehouse, N. L., V. M. Olson, C. G. Schwab, W. R. Chesbro, K. D. Cunningham, and T. Lykos. 1994. Improved techniques for dissociating particle-associated mixed ruminal microorganisms from ruminal digesta solids. J. Anim. Sci. 72:1335-1343.

Wixom, R. L., J. B. Shatton, and M. Strassman. 1960. Studies on a dehydrase in valine biosynthesis in yeast. J Biol Chem 235:128-131.

Wongtangtintharn, S., H. Oku, H. Iwasaki, and T. Toda. 2004. Effect of branched-chain fatty acids on fatty acid biosynthesis of human breast cancer cells. J Nutr Sci Vitaminol (Tokyo) 50:137-143.

Wu, Z. and D. Palmquist. 1991. Synthesis and biohydrogenation of fatty acids by ruminal microorganisms in vitro. J. Dairy Sci. 74:3035-3046.

Wu, Z., C. Wang, G. Zhang, Q. Liu, G. Guo, W. Huo, J. Zhang, Y. Zhang, C. Pei, and S. Zhang. 2019. Effects of pantothenic acid and folic acid supplementation on total tract digestibility coefficient, ruminal fermentation, microbial enzyme activity, microflora and urinary purine derivatives in dairy bulls. The Journal of Agricultural Science:1-8.

Yang, C. M. 2002. Response of forage fiber degradation by ruminal microorganisms to branched-chain volatile fatty acids, amino acids, and dipeptides. J. Dairy Sci. 85:1183-1190.

Yang, Z., S. Liu, X. Chen, H. Chen, M. Huang, and J. Zheng. 2000. Induction of apoptotic cell death and in vivo growth inhibition of human cancer cells by a saturated branched-chain fatty acid, 13-methyltetradecanoic acid. Cancer Res 60:505-509.

Yoder, P. S., X. Huang, I. A. Teixeira, J. P. Cant, and M. D. Hanigan. 2020. Effects of jugular infused methionine, lysine, and histidine as a group or leucine and isoleucine as a group on production and metabolism in lactating dairy cows. J. Dairy Sci. 103:2387-2404.

Yu, Z. and M. Morrison. 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques 36:808-812.

Zhang, H. L., Y. Chen, X. L. Xu, and Y. X. Yang. 2013. Effects of Branched-chain Amino Acids on In vitro Ruminal Fermentation of Wheat Straw. Asian-Australas J Anim Sci 26:523-528.

Zhang, X. M., R. F. Medrano, M. Wang, K. A. Beauchemin, Z. Y. Ma, R. Wang, J. N. Wen, B. A. Lukuyu, Z. L. Tan, and J. H. He. 2019. Corn oil supplementation enhances hydrogen use for biohydrogenation, inhibits methanogenesis, and alters fermentation pathways and the microbial community in the rumen of goats. J. Anim. Sci. 97:4999-5008.

Zhang, Y. L., Q. Liu, C. Wang, C. X. Pei, H. Y. Li, Y. X. Wang, W. Z. Yang, Y. S. Bai, Z. G. Shi, and X. N. Liu. 2015. Effects of supplementation of Simmental steers with 2-methylbutyrate on rumen microflora, enzyme activities and methane production. Anim. Feed Sci. Technol. 199:84-92.

Zhang, Y. M. and C. O. Rock. 2008. Membrane lipid homeostasis in bacteria. Nat. Rev. Microbiol. 6:222-233.

Zhu, K., K. H. Choi, H. P. Schweizer, C. O. Rock, and Y. M. Zhang. 2006. Two aerobic pathways for the formation of unsaturated fatty acids in Pseudomonas aeruginosa. Mol Microbiol 60:260-273.

Appendix A. Complete milk fatty acid profile

Table A.1. Complete fatty acid profile of milk produced by Jersey cows fed different combinations of branched-chain volatile fatty acids

Fatty Acid (g/100g)	Treatment[1]				SEM	Significance[2]					
	CON	MB	MB+IB	ISO		TRT	WK	PR	TRT × WK	TRT × PR	WK × PR
4:0	7.58	8.15	7.94	7.65	0.25	0.34	< 0.01	0.28	0.13	0.72	0.51
6:0	4.53	4.61	4.51	4.34	0.12	0.35	0.67	0.06	0.66	0.92	0.13
8:0 [3]	2.00	1.83	1.86	1.79	0.07	0.15	< 0.01	0.01	0.72	0.17	0.65
10:0 [3]	3.04	2.75	2.80	2.73	0.10	0.13	< 0.01	< 0.01	0.26	0.09	0.02
11:0	0.0307	0.0313	0.0322	0.0326	0.0027	0.96	0.03	0.02	0.16	0.77	0.01
12:0	2.81	2.80	2.75	2.65	0.11	0.75	0.03	< 0.01	0.31	0.81	< 0.01
13:0	0.0521	0.0566	0.0531	0.0522	0.0031	0.76	0.90	0.06	0.26	0.80	0.02
14:0 iso	0.0955	0.0969	0.0975	0.0914	0.0042	0.72	< 0.01	0.13	0.54	0.27	< 0.01
14:0	9.24	9.73	9.60	9.29	0.27	0.54	< 0.01	0.10	0.41	0.59	0.01
14:1 c9	0.525	0.607	0.570	0.545	0.029	0.15	< 0.01	0.21	0.66	0.15	0.09
15:0 iso	0.119	0.123	0.120	0.120	0.003	0.77	< 0.01	0.21	0.71	0.08	< 0.01
15:0 anteiso	0.264^{b}	0.291^{a}	0.264^{b}	0.273^{ab}	0.008	0.08	< 0.01	0.29	0.63	0.47	< 0.01
15:0	0.800	0.832	0.843	0.811	0.031	0.74	< 0.01	0.59	0.15	0.71	< 0.01
16:0 iso	0.228	0.230	0.232	0.226	0.008	0.95	< 0.01	0.07	0.94	0.91	< 0.01
16:0	33.2	34.1	33.9	32.6	0.6	0.25	< 0.01	0.26	0.90	0.18	0.08
17:0 iso	0.188	0.190	0.186	0.178	0.007	0.68	< 0.01	0.29	0.43	0.65	0.01
16:1 c9 [3]	0.738	0.811	0.809	0.720	0.040	0.19	< 0.01	0.28	0.17	0.61	0.35
17:0 anteiso	0.228	0.233	0.213	0.224	0.008	0.27	< 0.01	0.03	0.25	0.98	0.11
17:0	0.384	0.385	0.385	0.397	0.01	0.88	0.21	0.02	0.83	0.06	0.72
18:0	11.6^{ab}	10.4^{c}	10.8^{bc}	12.2^{a}	0.5	0.04	< 0.01	0.45	0.42	0.15	0.06
18:1 trans-5 and 4	0.0152	0.0105	0.0113	0.0007	0.0047	0.66	< 0.01	0.11	0.52	0.46	0.06
18:1 trans-6 to 8	0.223	0.216	0.216	0.211	0.009	0.75	< 0.01	0.61	0.64	0.07	0.98
18:1 trans-9	0.157	0.149	0.147	0.140	0.006	0.26	< 0.01	0.09	0.15	0.57	0.18

continued

Table A.1 continued

Fatty Acid (g/100g)	Treatment[1]				SEM	Significance[2]					
	CON	MB	MB+IB	ISO		TRT	WK	PR	TRT × WK	TRT × PR	WK × PR
18:1 trans-10	0.248	0.232	0.248	0.243	0.012	0.74	< 0.01	0.81	0.63	0.86	0.79
18:1 trans-11	0.666	0.680	0.697	0.688	0.035	0.91	< 0.01	0.25	0.86	0.05	0.87
18:1 trans-12	0.123	0.127	0.0942	0.0912	0.0302	0.74	0.03	0.05	0.47	0.16	0.12
18:1 cis-9	14.9	14.4	14.3	15.5	0.45	0.22	< 0.01	0.21	0.34	0.96	0.07
18:1 trans-15	0.370	0.342	0.365	0.377	0.015	0.29	< 0.01	0.63	0.29	0.07	0.04
18:1 cis-11	0.188	0.187	0.196	0.187	0.009	0.82	< 0.01	0.26	0.53	0.77	0.69
18:1 cis-12	0.0192	0.0124	0.0183	0.0221	0.0034	0.20	< 0.01	< 0.01	0.93	0.38	0.22
19:0 [3]	0.125	0.116	0.129	0.112	0.010	0.74	< 0.01	0.64	0.18	0.03	0.07
18:2 cis-9, cis-12	1.70	1.60	1.64	1.71	0.05	0.31	0.05	< 0.01	0.39	0.68	0.10
18:3 cis-6, cis-9, cis-12	0.0384	0.0282	0.0357	0.0434	0.0074	0.45	< 0.01	< 0.01	0.82	0.71	< 0.01
20:0	0.237	0.237	0.249	0.235	0.013	0.82	0.09	0.42	0.49	0.37	0.63
18:2 cis-9, trans-11 [3]	0.154	0.146	0.146	0.152	0.01	0.87	< 0.01	0.14	0.23	0.43	0.06
18:2 trans-10, cis-12	ND[4]	0.0007	ND	ND	0.0012	0.57	0.58	0.24	0.37	0.57	0.58
21:0 [3]	0.0725	0.0734	0.0691	0.0794	0.0112	0.93	< 0.01	0.36	0.62	0.91	0.63
Total Known	96.8	96.9	96.5	96.7	0.3	0.74	0.04	0.43	0.19	0.25	0.48

[a-c] Mean values in the same row with different superscripts differ ($P \leq 0.10$) between treatments.

[1] Treatments: Control (CON, no supplement); 2-methylbutyrate (MB, 12.3 mmol/kg DM); MB + isobutyrate (MB + IB, 7.7 and 12.6 mmol/kg DM, respectively); and MB + IB + isovalerate + valerate (ISO, 6.4, 7.3, 4.2, and 5.1 mmol/kg DM, respectively.

[2] Probability of treatment effects: TRT = effects of different combination of branched-chain volatile fatty acids and valerate, WK = effect of week, PR = effect of parity, TRT×WK = interaction between supplementation and week, TRT×PR = interaction between supplementation and parity, and WK×PR = interaction between week and parity.

[3] Interaction between supplementation, week, and parity ($P \leq 0.09$).

[4] ND indicates not detected in milk fatty acid analysis.

Appendix B. Aqueous hydrogen and gas production measurements of time point samples taken from dual flow continuous cultures

Table B.1. Concentration aqueous hydrogen [H_2(aq)], H_2 and CH_4 production rate in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation.

	HF[1]				LF				SEM	Significance[2]		
	-CO		+CO		-CO		+CO					
Item	- BCVFA	+ BCVFA	- BCVFA	+ BCVFA	- BCVFA	+ BCVFA	- BCVFA	+ BCVFA		Diet	CO	BCVFA
H_2(aq), µmol/L[3]	3.92	3.98	4.12	4.33	5.01	5.40	5.18	5.27	0.64	< 0.01	0.39	0.28
CH_4, mmol/h[3]	4.08	3.93	3.89	4.40	4.00	3.48	4.11	3.56	0.47	0.28	0.66	0.52
H_2, µmol/h[3]	7.60	5.60	9.15	5.40	14.0	23.4	18.4	19.2	7.68	0.03	0.94	0.83

[1] Treatments are high (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] P values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] The main effect of hour has $P \leq 0.01$.

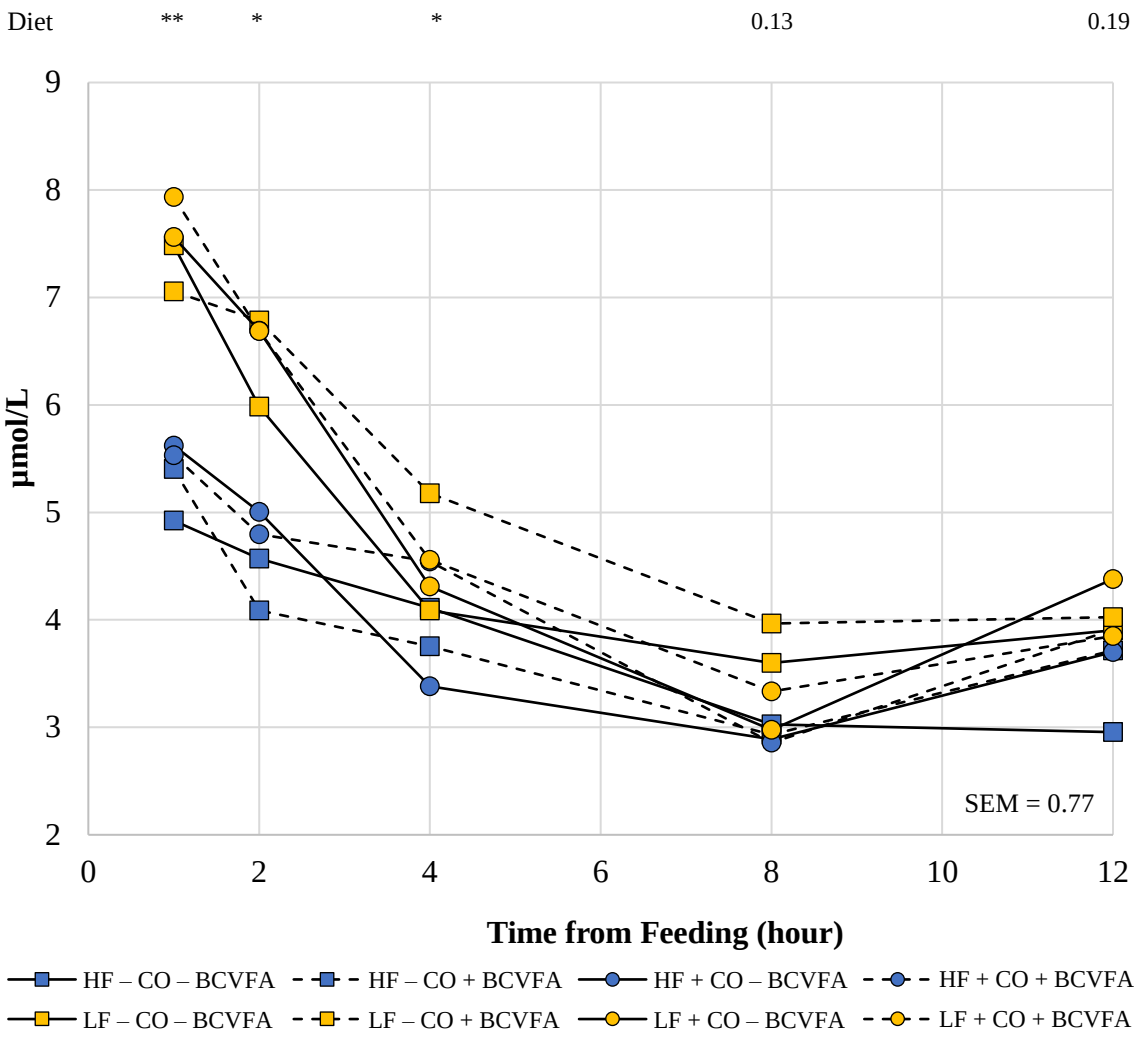

Figure B.1 Fermenter aqueous H_2 [H_2(aq)] concentration over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA).
Data are means from all 4 experimental periods taken on d 8 to 11 of the period. Main effects or interactions of treatments that had an interaction with hour ($P < 0.10$) were tested at each timepoint and are reported as: ** = $P \leq 0.05$, * = $0.05 \leq P \leq 0.10$, NS = $P > 0.20$. The SEM is pooled across treatment and is shown in the figure.

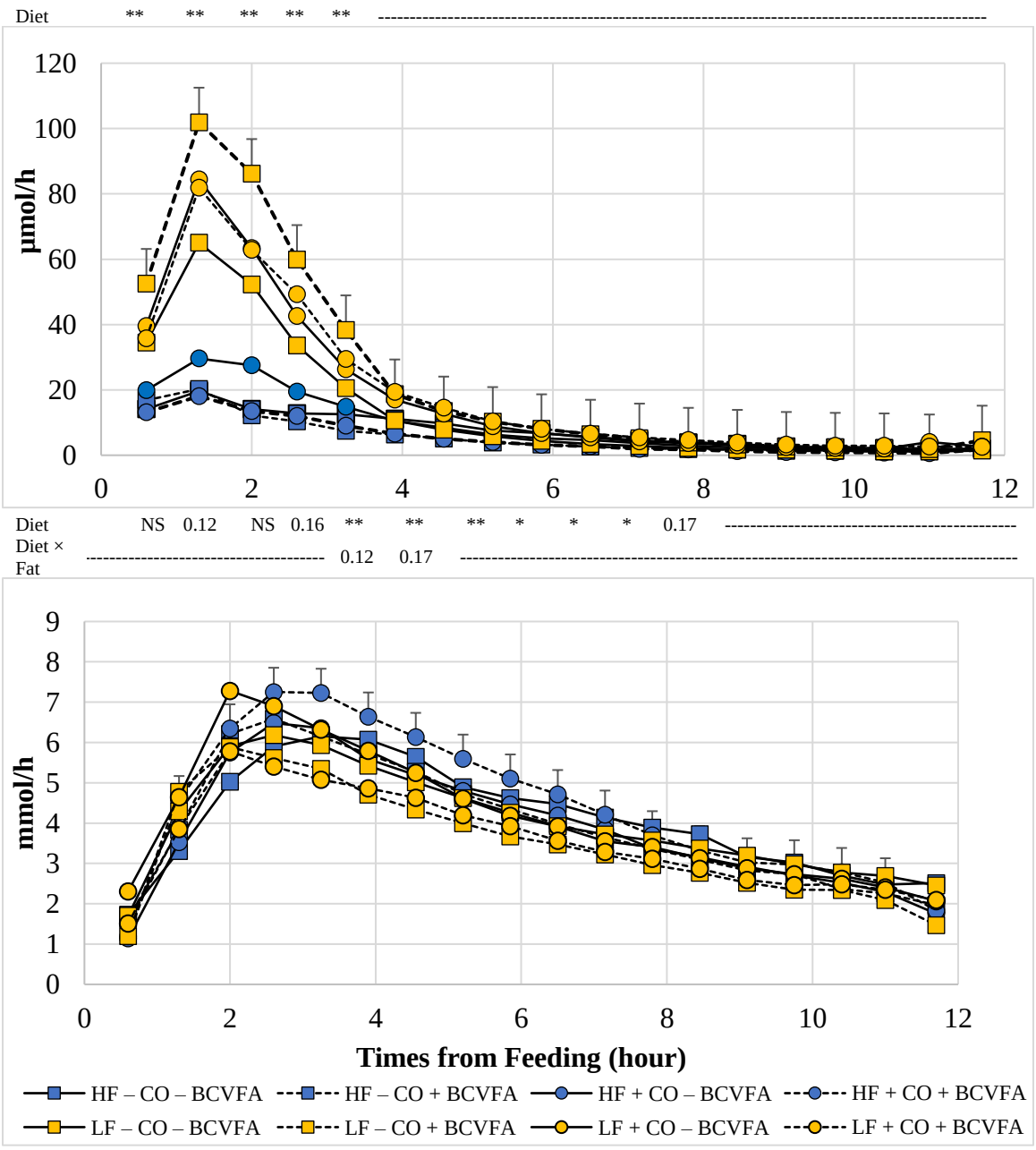

Figure B.2 Fermenter gaseous H_2 (top) and CH_4 (bottom) production rates over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA). Data are means from all 4 experimental periods taken on d 8 to 11 of the period.

Data are means from all 4 experimental periods taken on d 7 to 11 of the period. Main effects or interactions of treatments that had an interaction with hour ($P < 0.10$) were tested at each timepoint and are reported as: ** = $P \leq 0.05$, * = $0.05 \leq P \leq 0.10$, NS = $P > 0.20$. The SEM is pooled across treatment and is shown for 1 series in the figure.

Appendix C. Volatile fatty acids concentrations of time point samples taken from dual flow continuous cultures.

Table C.1. Concentration of VFA from in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation.

	HF[1]				LF				SEM	Significance[2]			
	-CO		+CO		-CO		+CO			Diet	CO	BCVFA	Diet × BCVFA
Item	- BCVFA	+ BCVFA	- BCVFA	+ BCVFA	- BCVFA	+ BCVFA	- BCVFA	+ BCVFA					
Concentration, mM													
Total VFA[3]	97.9	97.3	91.0	94.6	108	104	101	105	2.2	< 0.01	< 0.01	0.27	0.48
Acetate[3]	64.2	63.6	59.6	61.1	62.8	60.3	57.7	59.5	1.3	< 0.01	< 0.01	0.93	0.54
Propionate[3]	19.2	17.7	17.6	17.9	23.8	22.1	22.9	23.8	1.1	< 0.01	0.72	0.36	0.81
Acetate: propionate	3.37	3.59	3.37	3.42	2.67	2.77	2.55	2.58	0.13	< 0.01	0.16	0.21	0.67
Butyrate[3, 4]	10.6	10.1	10.1	10.1	15.7	14.4	14.7	15.5	0.6	< 0.01	0.68	0.36	0.97
Valerate[3]	1.48	1.48	1.44	1.46	1.94	1.77	1.84	1.73	0.07	< 0.01	0.06	0.02	0.01
Caproate[3]	0.526	0.640	0.513	0.544	1.11	1.02	1.01	0.90	0.076	< 0.01	0.11	0.78	0.09
Isobutyrate[3]	0.681	1.33	0.663	1.26	0.802	1.32	0.775	1.33	0.034	< 0.01	0.20	< 0.01	0.03
2-methylbutyrate[3]	0.561	1.18	0.551	1.11	0.937	1.35	0.865	1.30	0.054	< 0.01	0.15	< 0.01	0.01
Isovalerate[3]	0.589	1.25	0.580	1.19	0.845	1.38	0.819	1.36	0.039	< 0.01	0.24	< 0.01	0.05
Total BCVFA[3]	1.83	3.76	1.79	3.57	2.58	4.04	2.46	3.99	0.11	< 0.01	0.12	< 0.01	0.01

[1] Treatments are high (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] P values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] The main effect of hour has $P \leq 0.01$.

[4] Interaction of CO and BCVFA supplementation has $P = 0.08$.

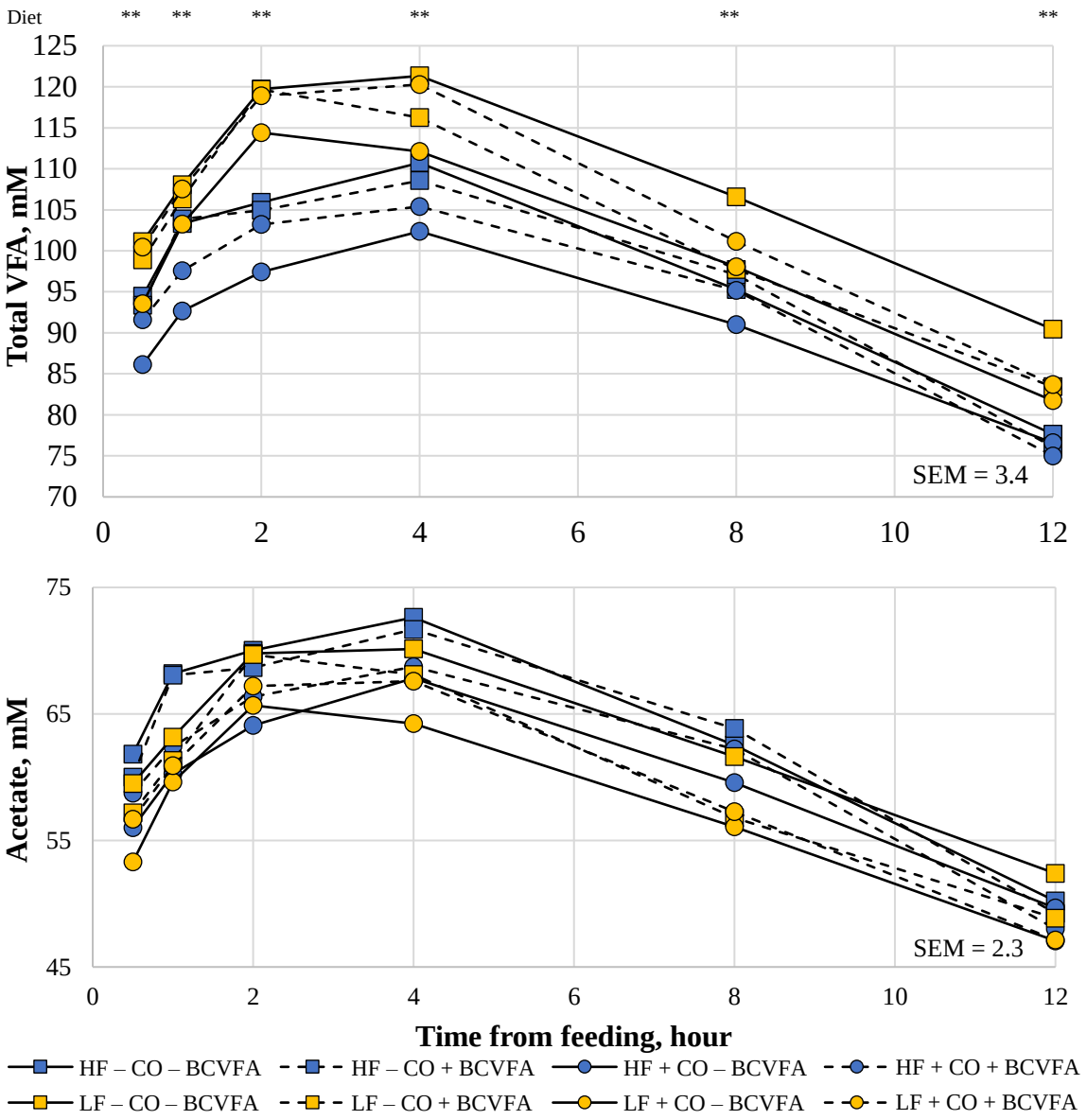

Figure C.1 Concentration of total VFA (top) and acetate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA).

Data are means from all 4 experimental periods taken on d 8 to 11 of the period. Main effects or interactions of treatments that had an interaction with hour ($P < 0.10$) were tested at each timepoint and are reported as: ** = $P \leq 0.05$, * = $0.05 \leq P \leq 0.10$, NS = $P > 0.20$. The SEM is pooled across treatment and is shown in the figure.

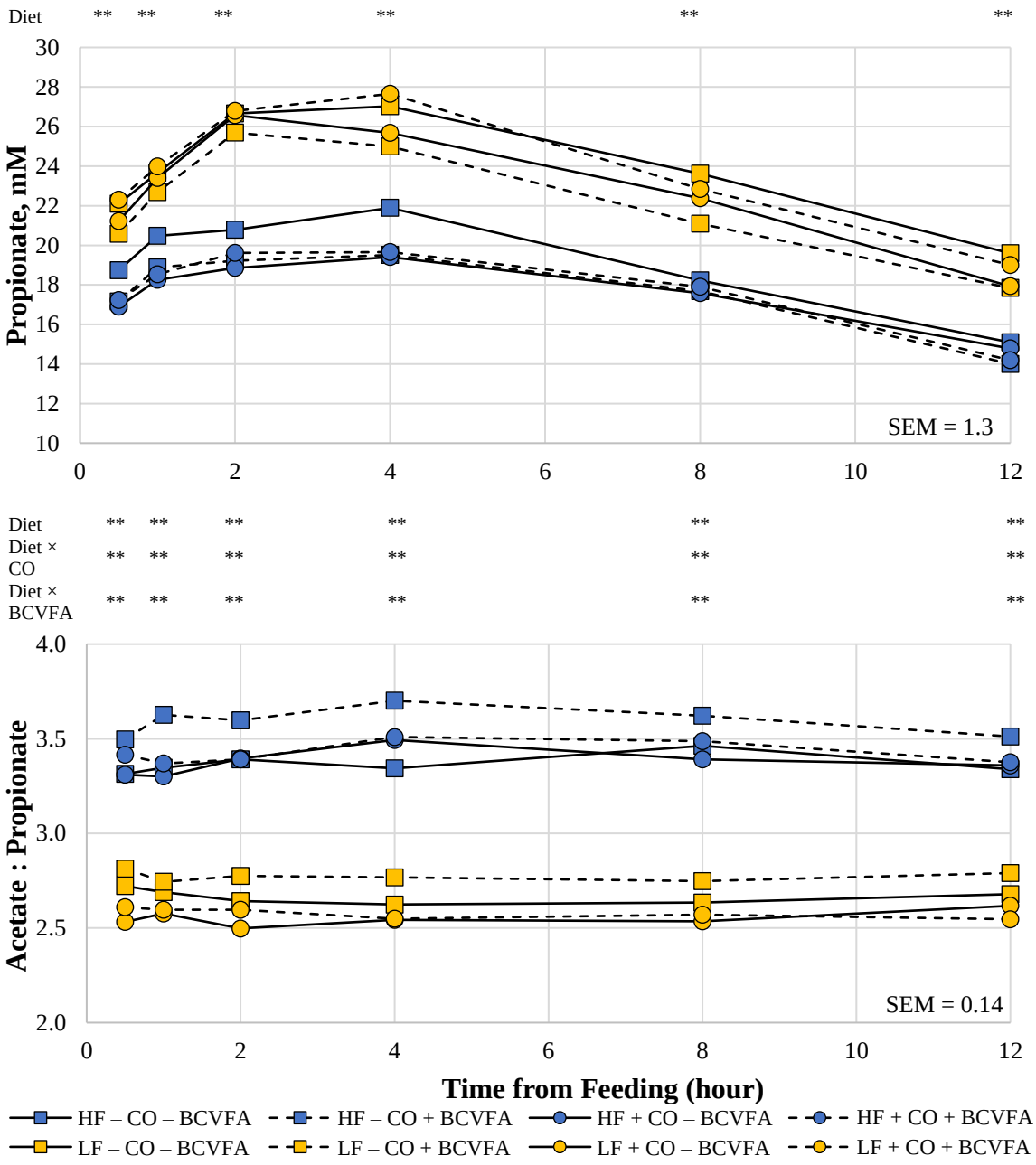

Figure C.2 Concentration of acetate (top) propionate (middle) and acetate: propionate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA).

Data are means from all 4 experimental periods taken on d 8 to 11 of the period. Main effects or interactions of treatments that had an interaction with hour ($P < 0.10$) were tested at each timepoint and are reported as: ** = $P \leq 0.05$, * = $0.05 \leq P \leq 0.10$, NS = $P > 0.20$. The SEM is pooled across treatment and is shown in the figure.

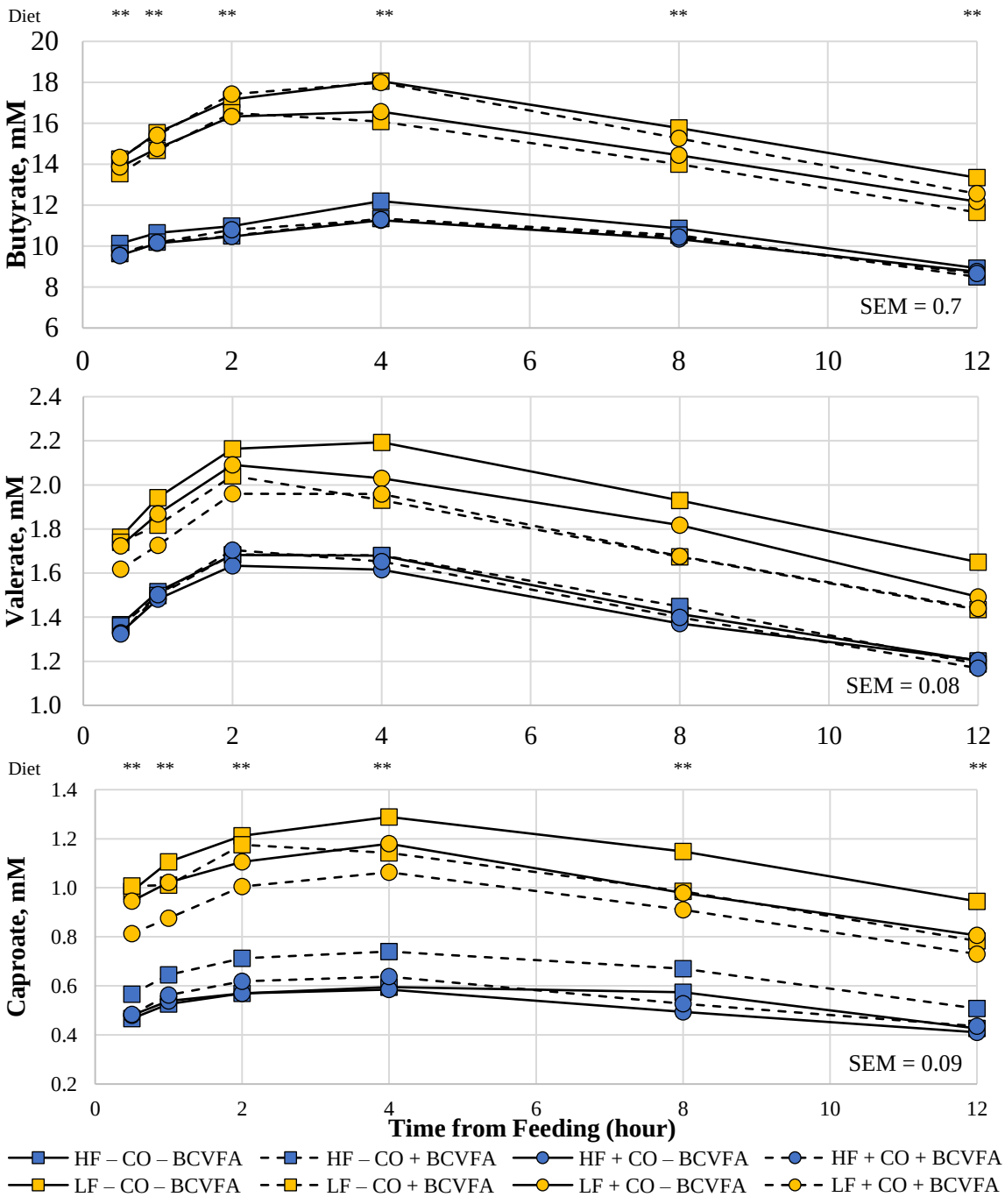

Figure C.3 Concentration of butyrate (top), valerate (middle), and caproate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA). Data are means from all 4 experimental periods taken on d 8 to 11 of the period. Main effects or interactions of treatments that had an interaction with hour ($P < 0.10$) were tested at each timepoint and are reported as: ** = $P \leq 0.05$, * = $0.05 \leq P \leq 0.10$, NS = $P > 0.20$. The SEM is pooled across treatment and is shown in the figure.

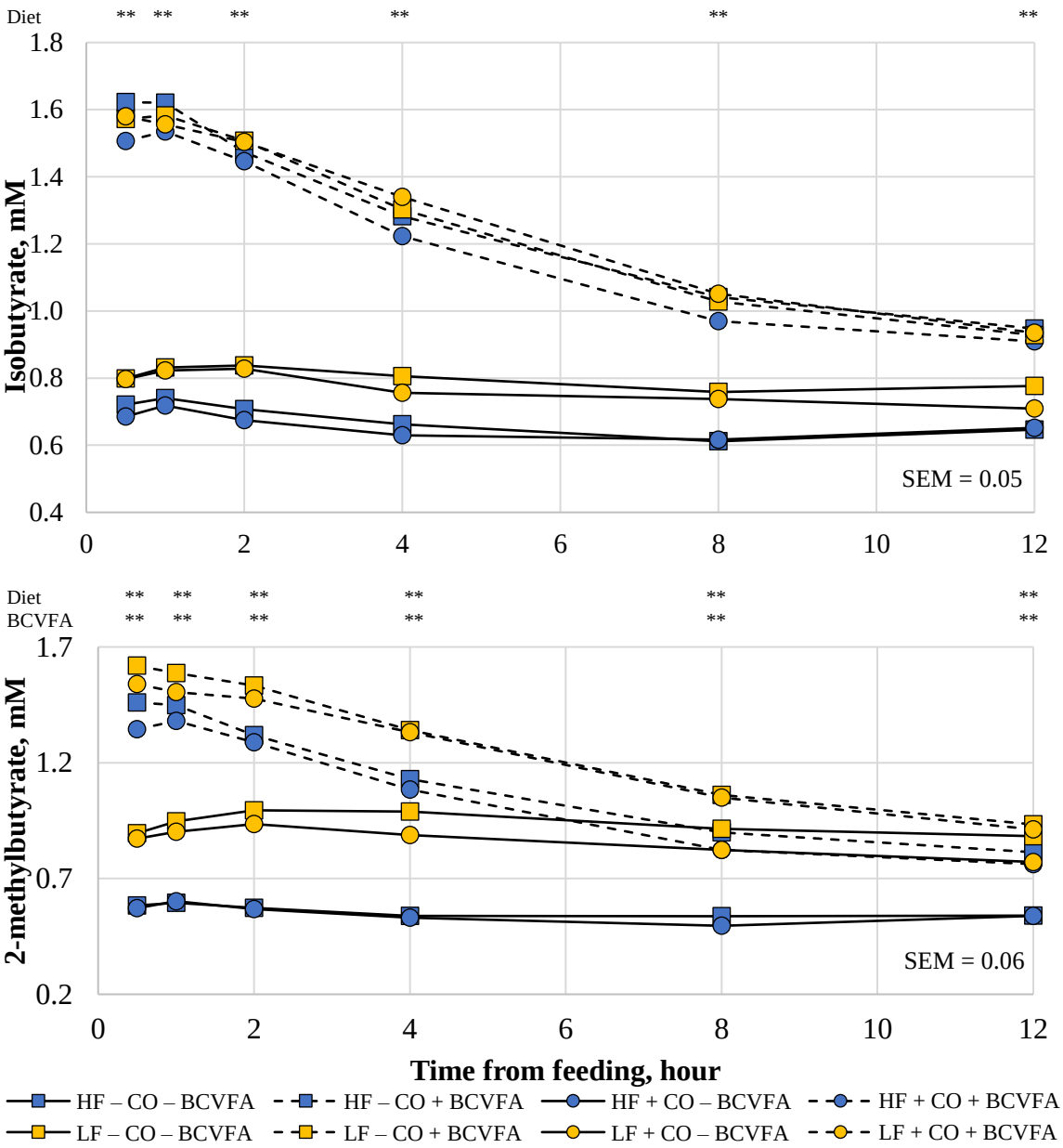

Figure C.4 Concentration of isobutyrate (top) and 2-methylbutyrate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA).
Data are means from all 4 experimental periods taken on d 8 to 11 of the period. Main effects or interactions of treatments that had an interaction with hour ($P < 0.10$) were tested at each timepoint and are reported as: ** = $P \leq 0.05$, * = $0.05 \leq P \leq 0.10$, NS = $P > 0.20$. The SEM is pooled across treatment and is shown in the figure.

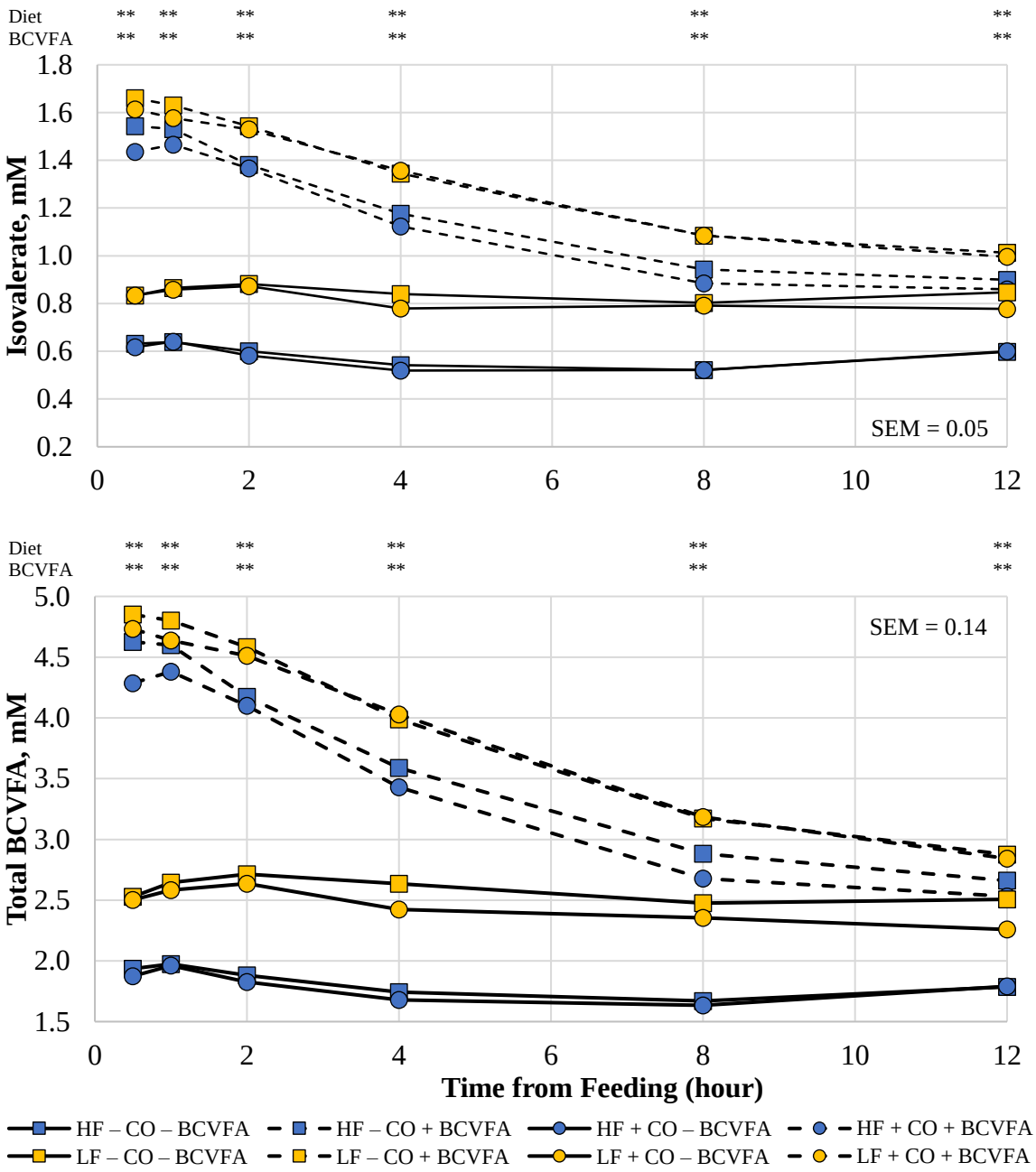

Figure C.5 Concentration of isovalerate (top) and total branched-chain VFA (BCVFA, bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental BCVFA (– BCVFA) or supplemental BCVFA (+ BCVFA).

Data are means from all 4 experimental periods taken on d 8 to 11 of the period. Main effects or interactions of treatments that had an interaction with hour ($P < 0.10$) were tested at each timepoint and are reported as: ** = $P \leq 0.05$, * = $0.05 \leq P \leq 0.10$, NS = $P > 0.20$. The SEM is pooled across treatment and is shown in the figure.

Appendix D. Molar proportions of time point samples taken from dual flow continuous cultures

Table D.1. Molar proportions of VFA from in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched chain VFA supplementation.

	HF[1]				LF				SEM	Significance[2]			
	-CO		+CO		-CO		+CO			Diet	CO	BCVFA	Diet × BCVFA
Item	- BCVFA	+ BCVFA	- BCVFA	+ BCVFA	- BCVFA	+ BCVFA	- BCVFA	+ BCVFA					
VFA, mol/100 mol													
Acetate[3]	65.5	65.2	65.3	64.4	58.0	58.1	57.3	56.5	0.6	< 0.01	0.00	0.08	0.67
Propionate	19.6	18.3	19.4	18.9	22.1	21.4	22.7	22.5	0.9	< 0.01	0.36	0.21	0.68
Butyrate[3]	0.704	1.37	0.738	1.34	0.749	1.27	0.784	1.26	0.027	0.27	0.74	< 0.01	< 0.01
Valerate[3]	10.9	10.5	11.1	10.8	14.6	13.9	14.7	14.7	0.6	< 0.01	0.14	0.17	0.90
Caproate	0.582	1.21	0.612	1.18	0.878	1.30	0.876	1.24	0.050	< 0.01	0.56	< 0.01	< 0.01
Isobutyrate[3]	0.611	1.29	0.649	1.26	0.792	1.34	0.832	1.30	0.031	< 0.01	0.89	< 0.01	< 0.01
2-Methylbutyrate[3]	1.51	1.53	1.58	1.54	1.80	1.72	1.85	1.65	0.05	< 0.01	0.43	< 0.01	< 0.01
Isovalerate[3, 4]	0.542	0.660	0.561	0.577	1.04	0.992	1.01	0.861	0.077	< 0.01	0.28	0.76	0.10
Total BCVFA[3]	1.90	3.87	2.00	3.78	2.42	3.91	2.49	3.80	0.09	< 0.01	0.88	< 0.01	< 0.01

[1] Treatments are high (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] P values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] The main effect of hour has $0.01 \leq P \leq 0.07$.

[4] Interaction of CO and BCVFA supplementation has $P = 0.08$.

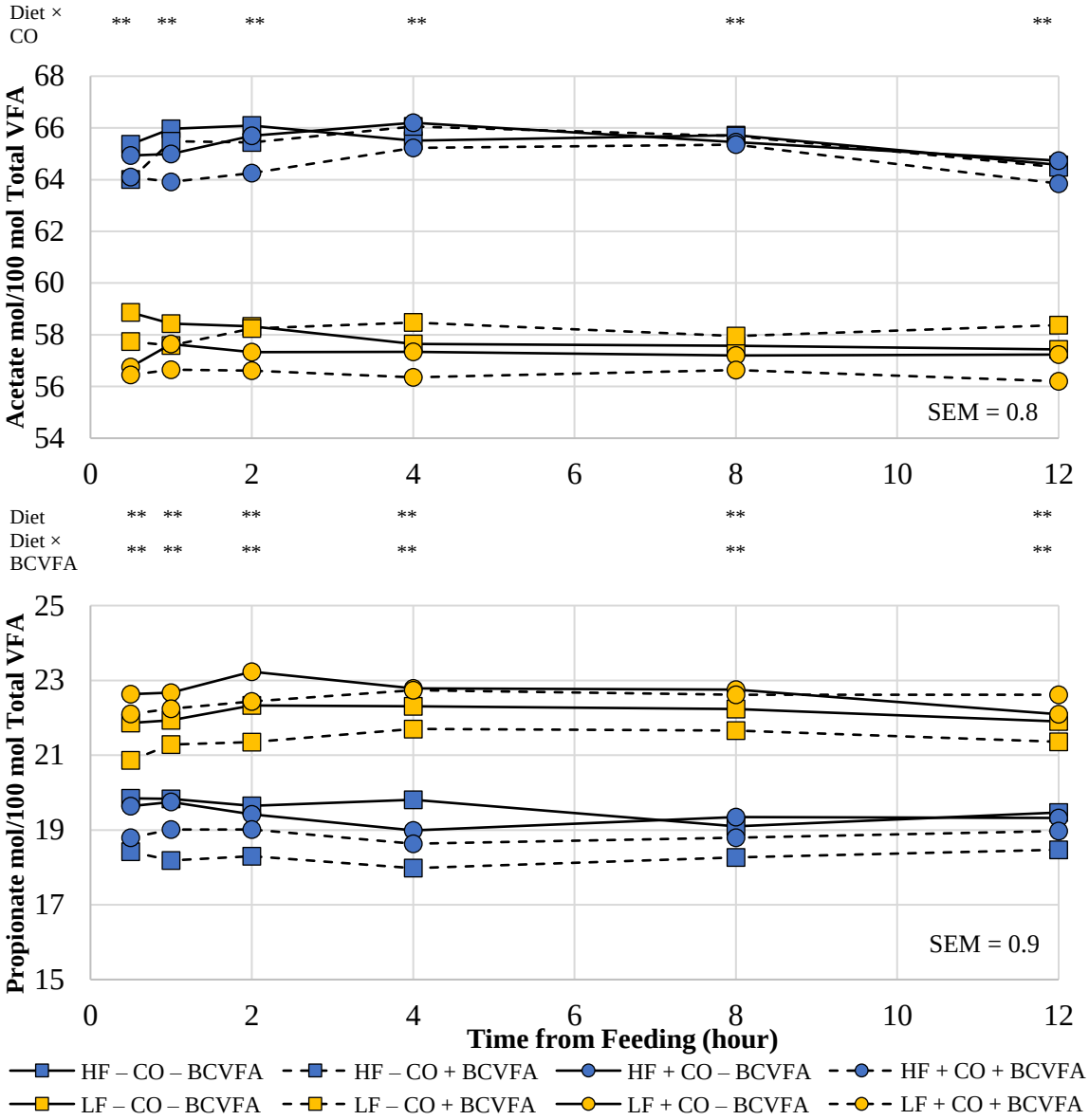

Figure D.1 Molar proportion of acetate (top) and propionate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA).

Data are means from all 4 experimental periods taken on d 8 to 11 of the period. Main effects or interactions of treatments that had an interaction with hour ($P < 0.10$) were tested at each timepoint and are reported as: ** = $P \leq 0.05$, * = $0.05 \leq P \leq 0.10$, NS = $P > 0.20$. The SEM is pooled across treatment and is shown in the figure.

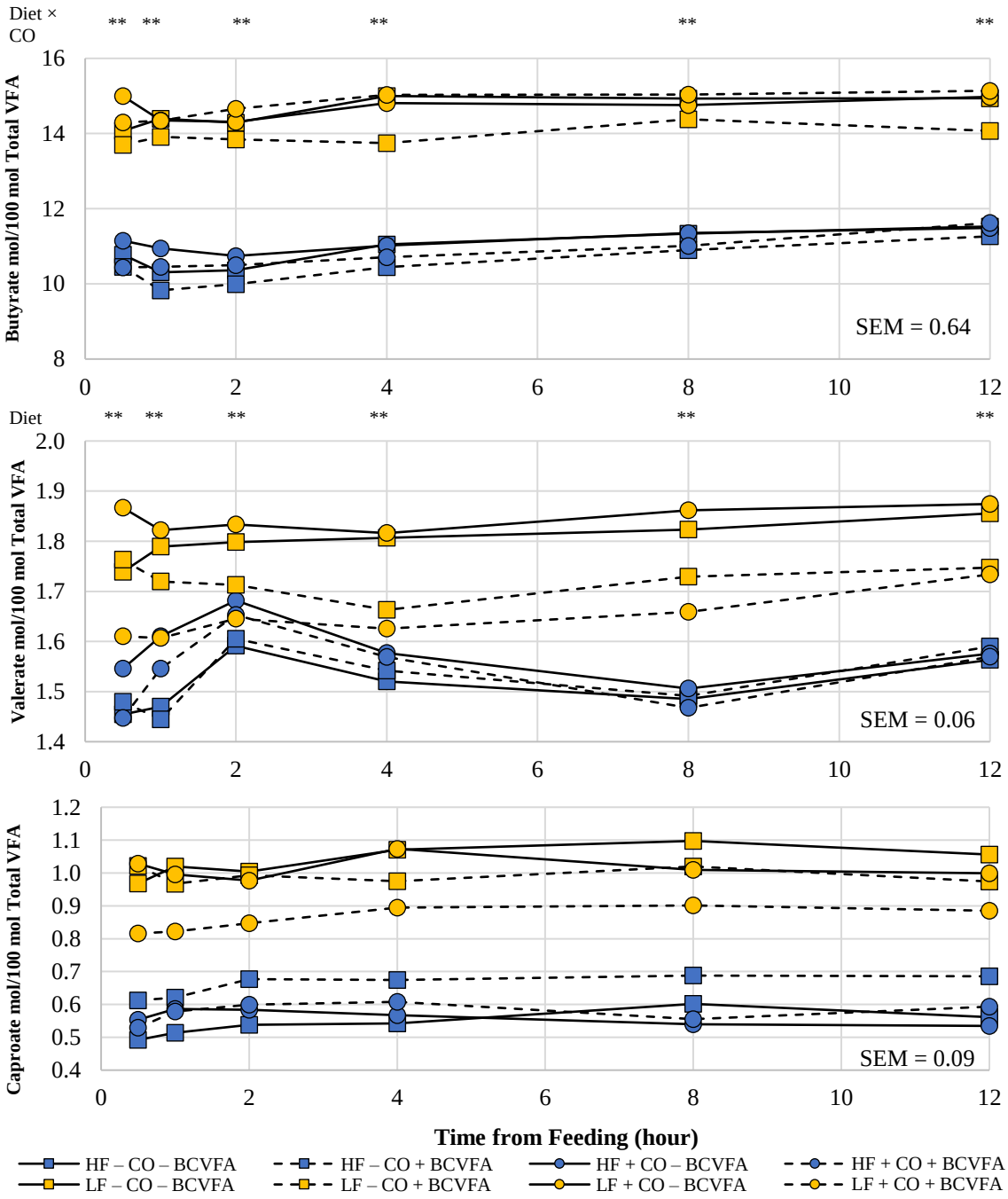

Figure D.2 Molar proportion of butyrate (top), valerate (middle), and caproate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA). Data are means from all 4 experimental periods taken on d 8 to 11 of the period. Main effects or interactions of treatments that had an interaction with hour ($P < 0.10$) were tested at each timepoint and are reported as: ** = $P \leq 0.05$, * = $0.05 \leq P \leq 0.10$, NS = $P > 0.20$. The SEM is pooled across treatment and is shown in the figure.

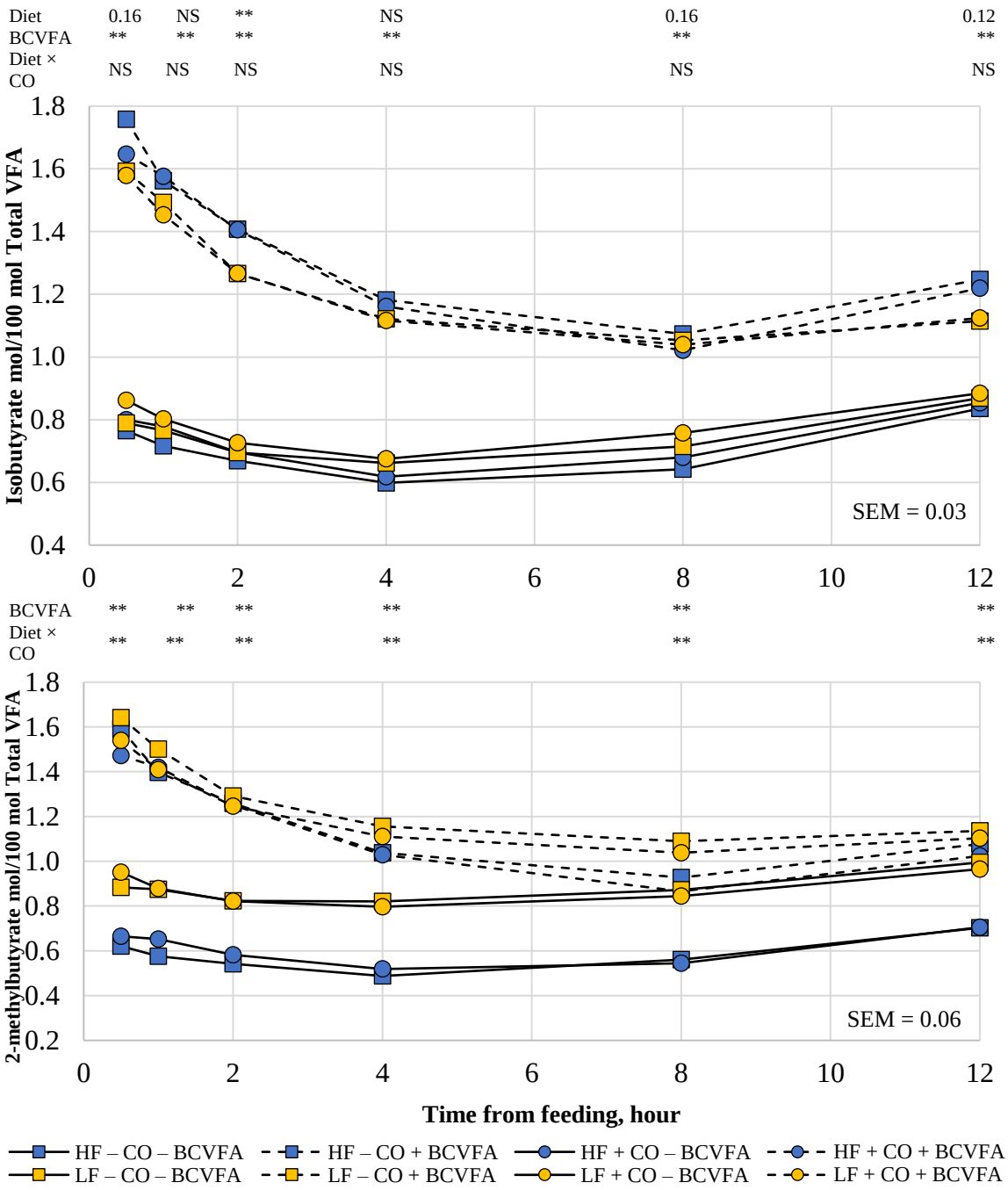

Figure D.3 Molar proportion of isobutyrate (top) and 2-methylbutyrate (bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental branched-chain VFA (– BCVFA) or supplemental BCVFA (+ BCVFA).
Data are means from all 4 experimental periods taken on d 8 to 11 of the period. Main effects or interactions of treatments that had an interaction with hour ($P < 0.10$) were tested at each timepoint and are reported as: ** = $P \leq 0.05$, * = $0.05 \leq P \leq 0.10$, NS = $P > 0.20$. The SEM is pooled across treatment and is shown in the figure.

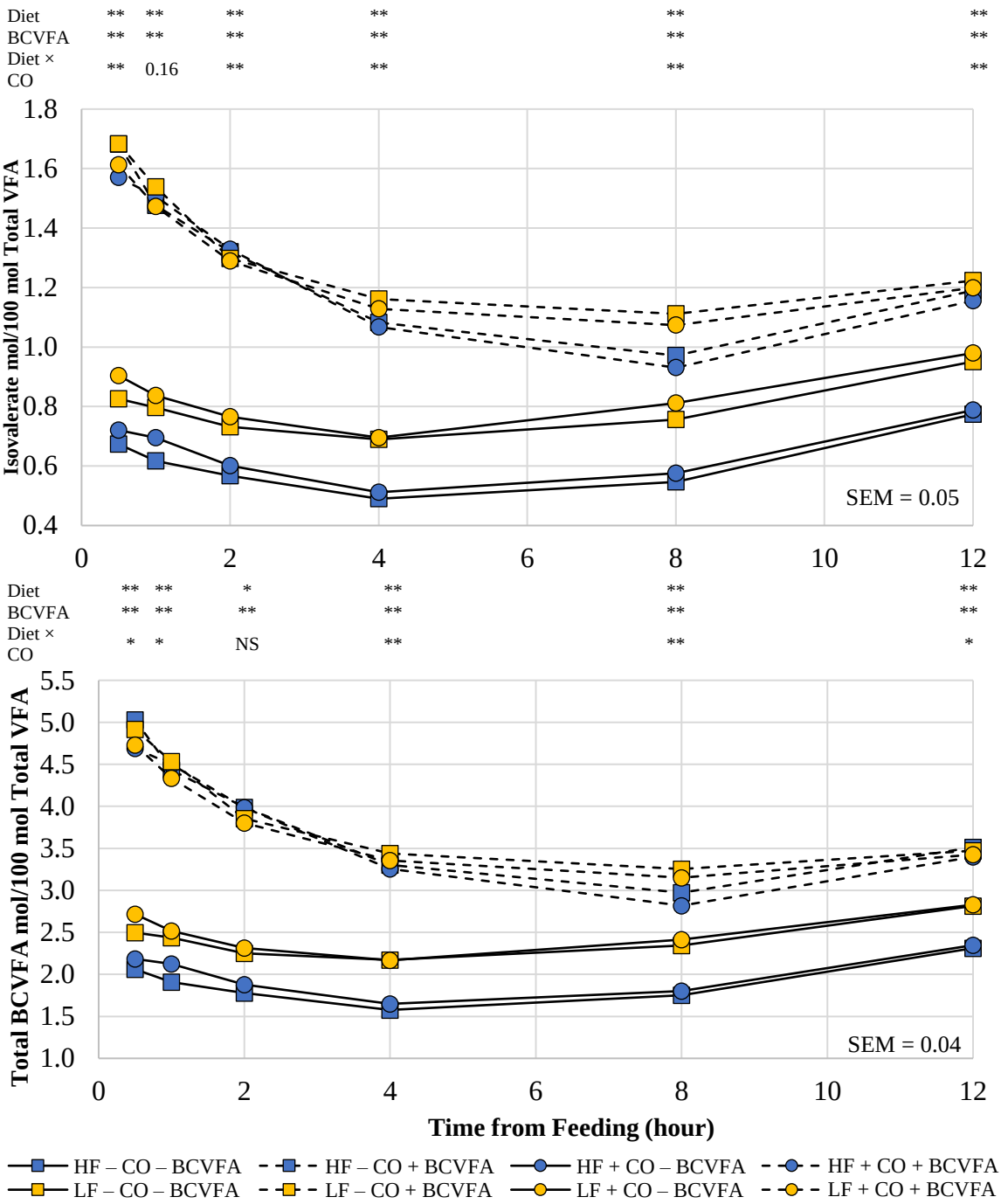

Figure D.4 Molar proportion of isovalerate (top) and total branched-chain VFA (BCVFA, bottom) over time post-feeding for continuous cultures that had treatments of high (HF) or low forage (LF) diets, no supplemental fat (– CO) or supplemental corn oil (+ CO), and either no supplemental BCVFA (– BCVFA) or supplemental BCVFA (+ BCVFA).

Data are means from all 4 experimental periods taken on d 8 to 11 of the period. Main effects or interactions of treatments that had an interaction with hour ($P < 0.10$) were tested at each timepoint and are reported as: ** = $P \leq 0.05$, * = $0.05 \leq P \leq 0.10$, NS = $P > 0.20$. The SEM is pooled across treatment and is shown in the figure.

Appendix E. Completed bacterial fatty acids and fatty aldehyde flows and isotope recovery tables from dual flow continuous cultures

Table E.1. Bacterial fatty acids (FA) flows in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation.

	HF[1]				LF				SEM	Significance[2]		
	– CO		+ CO		– CO		+ CO					
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA
Total FA flow, mg/d												
11:0 *iso*	0.0603	0.0755	0.0615	0.0505	0.0366	0.0730	0.0428	0.0870	0.0337	0.90	0.96	0.21
11:0 *anteiso*	0.0301	0.0313	0.0296	0.0495	0.0330	0.0509	0.0367	0.0488	0.0388	0.58	0.71	0.33
11:0	0.266	0.279	0.280	0.336	0.215	0.225	0.279	0.325	0.075	0.49	0.18	0.46
12:0 *iso*	0.129	0.180	0.136	0.138	0.0406	0.0940	0.0517	0.0722	0.042	< 0.01	0.55	0.11
12:0	4.10	4.79	5.74	4.26	6.61	6.03	7.07	7.57	1.17	< 0.01	0.20	0.71
13:0 *iso*	1.97	2.79	2.86	2.08	2.12	2.52	2.43	3.12	0.30	0.53	0.17	0.16
13:0 *anteiso*	0.307	0.378	0.460	0.385	0.250	0.314	0.305	0.319	0.068	0.07	0.22	0.67
13:0	2.18	2.27	2.61	1.96	2.37	1.85	2.22	2.06	0.23	0.42	0.79	0.07
14:0 *iso*	8.96	11.2	9.41	5.92	6.81	7.65	5.44	6.00	1.5	0.01	0.02	0.96
14:0	34.1	33.8	42.7	27.3	48.5	38.5	49.1	47.8	5.5	< 0.01	0.38	0.06
15:0 *iso*	21.5	23.8	31.4	20.8	14.6	15.5	15.4	19.2	3.8	0.01	0.28	0.74
15:0 *anteiso*	41.0	40.6	47.8	33.1	42.0	32.9	36.2	39.8	5.7	0.35	0.97	0.11
15:0	39.3	37.4	37.5	28.9	33.0	26.3	25.4	24.7	5.5	< 0.01	0.03	0.04
16:0 *iso*	7.22	9.73	8.84	7.54	4.25	5.36	3.66	4.12	1.24	< 0.01	0.34	0.27
16:0	289	255	259	219	300	245	296	302	33	0.13	0.86	0.13
17:0 *iso*	4.88	6.08	4.78	4.39	3.09	3.72	2.43	3.78	0.68	< 0.01	0.16	0.10
16:1 *cis*-7	1.46	1.54	1.94	1.14	0.97	0.79	0.95	2.02	0.49	0.34	0.37	0.90
16:1 *cis*-9	2.24	2.28	3.15	2.74	3.43	2.28	3.28	3.35	0.67	0.25	0.17	0.38
17:0 *anteiso*	6.75	7.91	7.24	5.75	5.36	5.52	4.41	5.97	0.94	< 0.01	0.25	0.46
16:1 *trans*-3	0.540	1.28	0.459	0.717	0.873	1.26	0.736	0.422	0.396	0.76	0.10	0.27

continued

Table E.1 continued.

Item	HF[1]				LF				SEM	Significance[2]		
	– CO		+ CO		– CO		+ CO			Diet	CO	BCVFA
	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA				
Total FA flow, mg/d												
17:0	9.83	9.62	7.94	6.83	8.46	6.32	6.86	7.30	1.17	0.07	0.07	0.28
18:0 *iso*[4]	0.819	1.16	0.524	0.745	0.913	0.915	0.591	0.568	0.131	0.43	< 0.01	0.11
18:0	401	488	369	340	421	348	403	425	59	0.99	0.46	0.96
18:1 *trans*-4	0.372	0.685	0.958	0.737	0.948	0.730	1.02	1.23	0.147	0.01	0.01	0.83
18:1 *trans*-5	0.267	0.511	0.528	0.459	0.515	0.528	0.731	0.823	0.097	< 0.01	0.01	0.25
18:1 *trans*-6 and -8	3.567	5.641	6.304	6.294	6.615	7.054	8.538	11.481	1.506	0.01	0.03	0.19
18:1 *trans*-9	2.26	3.23	4.23	4.29	3.45	4.14	4.42	7.58	1.17	0.11	0.04	0.15
18:1 *trans*-10[4]	4.10	6.05	6.05	7.42	7.11	5.74	8.98	5.27	1.63	0.36	0.22	0.64
18:1 *trans*-11	32.7	49.4	53.4	51.9	47.6	48.6	57.1	79.8	10.5	0.11	0.03	0.17
18:1 *trans*-12	4.15	6.12	7.74	7.54	6.34	6.53	8.55	11.82	1.34	0.04	< 0.01	0.15
18:1 *cis* 9	35.7	52.9	50.4	51.2	48.8	51.0	53.1	75.3	7.1	0.06	0.04	0.04
18:1 *trans*-15 and *cis*-11	18.8	23.6	26.5	24.3	50.3	42.6	51.2	61.3	8.4	< 0.01	0.20	0.81
18:1 *cis*-12	3.63	5.04	7.23	7.02	5.58	6.21	6.87	11.3	1.07	0.03	< 0.01	0.05
18:1 *cis*-13[4]	0.382	0.328	0.554	0.563	0.490	0.522	0.549	0.955	0.102	0.02	< 0.01	0.17
18:1 *trans*-16 and *cis*-14	2.82	4.87	4.25	4.45	3.90	3.90	4.88	5.91	0.82	0.30	0.07	0.13
18:1 *cis*-15	0.535	0.856	0.787	0.741	0.644	0.606	0.816	0.889	0.150	0.92	0.10	0.37
19:0	7.82	11.1	8.65	7.64	6.07	7.00	6.07	7.38	0.95	0.01	0.42	0.12
18:1 *cis*-16	0.498	0.702	0.613	0.823	0.530	0.663	0.870	1.131	0.211	0.25	0.04	0.10
18:2 t9, *cis*-12	0.152	1.277	0.377	0.775	0.381	0.542	0.556	0.638	0.258	0.47	0.99	0.01
18:2 *trans*-11, *cis*-15	0.659	0.769	0.707	0.585	0.534	0.253	0.583	0.491	0.283	0.19	0.81	0.55
18:2 *cis*-9, *cis*-12[4]	12.1	17.9	14.6	15.7	18.1	19.8	17.1	21.4	2.4	0.03	0.90	0.07
20:0[5]	5.40	7.81	4.04	3.72	3.52	3.44	3.56	2.69	0.84	< 0.01	0.02	0.62

continued

Table E.1 continued.

	HF[1]				LF				SEM	Significance[2]		
	– CO		+ CO		– CO		+ CO					
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA
FA Partial Sums, mg/d												
∑16:1[3]	5.75	8.06	8.06	5.81	6.29	5.74	5.70	7.18	1.14	0.37	0.76	0.75
∑18:1	110	160	170	168	183	179	208	275	30	0.01	0.03	0.16
∑BCFA[3,6]	93.6	104	114	81.0	79.5	74.7	71.0	83.1	12.9	0.01	0.91	0.60
∑ *iso* even BCFA	17.1	22.3	18.9	14.3	12.0	14.0	9.7	10.8	2.7	< 0.01	0.04	0.50
∑ *iso* odd BCFA	28.4	32.7	39.1	27.4	19.8	21.8	20.2	26.2	4.5	< 0.01	0.40	0.96
∑ *anteiso* BCFA[3]	48.1	48.9	55.6	39.2	47.7	38.8	41.0	46.2	6.6	0.204	0.91	0.18
∑ECFA[7]	734	789	681	595	780	642	759	786	87	0.49	0.60	0.56
∑OCFA[3, 8]	59.1	60.3	56.7	45.3	49.9	41.4	40.5	41.4	7.1	< 0.01	0.04	0.16

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] *P* values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] Interaction of Diet × CO × BCVFA supplementation ($0.02 \leq P \leq 0.10$).

[4] Interaction of Diet × BCVFA supplementation has ($0.04 \leq P \leq 0.09$).

[5] Interaction of Diet × CO supplementation has $P = 0.05$.

[6] Total branched-chain fatty acids.

[7] Total even straight-chain fatty acids.

[8] Total odd straight-chain fatty acids.

Table E.2. Bacterial fatty aldehyde (ALD) flows in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation.

	HF[1]				LF					Significance[2]			
	– CO		+ CO		– CO		+ CO		SEM				
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA	Diet × CO
ALD flow, mg/d													
11:0 *iso*[3, 4]	0.122	0.081	0.045	0.067	0.051	0.068	0.071	0.089	0.017	0.27	0.13	0.60	0.00
11:0 *anteiso*	0.052	0.025	0.048	0.040	0.032	0.029	0.051	0.052	0.041	0.98	0.12	0.28	0.37
11:0	0.167	0.098	0.080	0.113	0.029	0.094	0.066	0.132	0.037	0.21	0.98	0.39	0.18
12:0 *iso*[4]	2.90	1.43	1.03	1.48	1.33	1.39	1.34	1.36	0.37	0.10	0.04	0.26	0.04
12:0[3]	1.61	0.94	0.65	0.91	1.30	1.09	1.32	1.22	0.23	0.16	0.15	0.22	0.06
13:0 *iso*[3]	1.98	1.27	1.17	1.33	1.33	1.18	1.58	1.56	0.21	0.82	0.79	0.13	0.01
13:0 *anteiso*	3.95	2.27	2.22	2.61	3.65	2.88	3.66	3.25	0.56	0.10	0.48	0.10	0.22
13:0	1.16	0.58	0.57	0.63	0.83	0.72	0.84	0.78	0.16	0.59	0.26	0.11	0.15
14:0 *iso*	10.1	8.03	6.14	7.52	8.87	8.39	9.75	9.56	1.4	0.16	0.46	0.67	0.06
14:0	3.66	3.57	2.37	2.78	3.72	3.11	4.14	3.92	0.57	0.07	0.53	0.69	0.02
15:0 *iso*	2.76	2.67	2.01	2.52	2.22	1.98	2.49	2.72	0.37	0.52	0.91	0.65	0.04
15:0 *anteiso*	7.88	7.80	5.10	6.74	9.20	7.09	9.63	8.75	1.35	0.05	0.60	0.67	0.09
15:0	1.86	1.92	1.18	1.53	1.53	1.31	1.57	1.38	0.24	0.32	0.17	0.99	0.10
16:0 *iso*	1.44	1.52	0.84	1.18	0.71	0.86	0.76	0.83	0.18	< 0.01	0.07	0.19	0.05
16:0	8.27	8.54	6.03	8.09	10.9	9.68	12.6	12.4	1.98	< 0.01	0.69	0.85	0.12
17:0 *iso*[4]	0.295	0.206	0.113	0.200	0.210	0.334	0.342	0.198	0.072	0.14	0.28	0.90	0.30
17:0 *anteiso*	0.207	0.255	0.101	0.196	0.082	0.124	0.182	0.120	0.040	0.03	0.53	0.26	0.03
17:0	1.25	0.906	0.807	1.55	2.23	2.09	2.68	2.89	0.548	< 0.01	0.16	0.63	0.34
18:0 *iso*	0.303	0.18	0.153	0.157	0.193	0.119	0.214	0.070	0.054	0.21	0.20	0.04	0.35
18:0	0.253	0.143	0.238	0.334	0.301	0.191	0.329	0.281	0.111	0.66	0.34	0.57	0.84

continued

Table E.2 continued

	HF[1]				LF				SEM	Significance[2]			
	– CO		+ CO		– CO		+ CO						
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA	Diet × CO
Partial Sums, mg/d													
∑16:1	1.49	0.979	0.864	1.05	1.15	1.28	1.16	1.21	0.296	0.57	0.40	0.86	0.51
∑18:1	5.80	4.68	3.69	4.05	5.96	5.01	6.31	5.91	1.39	0.09	0.59	0.45	0.16
∑BCALD[5]	39.5	31.1	23.4	29.5	33.2	29.4	35.9	34.1	4.8	0.45	0.39	0.51	0.05
∑ *iso* even BCALD	17.1	12.9	9.6	12.0	12.5	12.1	13.5	13.2	2.1	0.97	0.22	0.62	0.04
∑ *iso* odd BCALD	7.65	6.04	4.98	5.87	5.44	5.00	6.16	6.23	0.81	0.35	0.63	0.55	0.02
∑ *anteiso* BCALD	14.8	12.2	8.9	11.7	15.3	12.3	16.2	14.6	2.2	0.07	0.57	0.45	0.10
∑ECALD[6]	13.8	13.2	9.3	12.1	16.2	14.1	18.4	17.8	2.7	0.01	0.95	0.93	0.08
∑OCALD[7]	4.43	3.51	2.63	3.82	4.62	4.22	5.16	5.18	0.86	0.02	> 0.99	0.95	0.12

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] *P* values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] Interaction of CO × BCVFA supplementation ($0.06 \leq P \leq 0.09$).

[4] Interaction of Diet × CO × BCVFA interaction ($0.02 \leq P \leq 0.07$).

[5] Branched-chain fatty aldehydes.

[6] Even straight-chain aldehydes.

[7] Odd straight-chain aldehydes.

Table E.3. Dose recovery in bacterial fatty acids (FA) in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation.

Total ^{13}C recovery in bacterial FA C flow, µg/mg of ^{13}C dosed[3]	HF[1]		LF		SE	Significance[2]		
	– CO	+ CO	– CO	+ CO		Diet	CO	Diet × CO
11:0 *iso*	0.00096	0.00017	0.00085	0.00087	0.00035	0.41	0.45	0.20
11:0 *anteiso*	0.00035	0.00023	0.00053	0.00050	0.00022	0.41	0.77	0.83
12:0 *iso*	0.00438	0.00299	0.00151	0.00120	0.00091	0.08	0.49	0.39
13:0 *iso*	0.0621	0.0444	0.0518	0.0604	0.0058	0.49	0.59	0.03
13:0 *anteiso*	0.00800	0.00786	0.00542	0.00542	0.00069	0.08	0.93	0.92
14:0 *iso*	0.239	0.130	0.147	0.106	0.036	0.23	0.14	0.35
15:0 *iso*	0.401	0.336	0.222	0.273	0.041	0.08	0.90	0.17
15:0 *anteiso*	0.697	0.573	0.432	0.497	0.076	0.02	0.51	0.03
16:0 *iso*	0.199	0.159	0.087	0.062	0.026	0.03	0.15	0.65
17:0 *iso*	0.0660	0.0487	0.0303	0.0352	0.0038	0.02	0.19	0.07
17:0 *anteiso*	0.0847	0.0749	0.0373	0.0407	0.0166	0.01	0.73	0.17
18:0 *iso*	0.0157	0.0074	0.0102	0.0056	0.0013	0.12	0.05	0.26

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil).

[2] *P* values for main effect means for diet (HF vs LF), CO, Diet × CO interactions. Only the branched-chain VFA (BCVFA) treatments were dosed with ^{13}C. The model also included a covariate (BCVFA mmol/d production) for the differences between diet for RDP and feed AA profile.

[3] The ^{13}C dose was provided as BCVFA (5 mg/d of ^{13}C from each isobutyrate, 2-methylbutyrate, and isovalerate).

Table E.4. Recovered dose distribution in bacterial fatty acids (FA) in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation.

Distribution of ^{13}C in bacterial lipid outflow, %[3]	HF[1]		LF		SE	Significance[2]		
	– CO	+ CO	– CO	+ CO		Diet	CO	Diet × CO
11:0 *iso*	0.00694	0.00390	0.00377	0.00651	0.00266	0.92	0.96	0.38
11:0 *anteiso*	0.00268	0.00278	0.00290	0.00427	0.00167	0.59	0.74	0.68
12:0 *iso*	0.0273	0.0216	0.0078	0.0128	0.0055	0.09	0.94	0.35
13:0 *iso*	0.404	0.340	0.477	0.578	0.035	0.01	0.74	0.04
13:0 *anteiso*	0.0513	0.0569	0.0514	0.0551	0.0058	0.88	0.46	0.87
14:0 *iso*	1.53	0.952	1.06	1.07	0.21	0.41	0.37	0.22
15:0 *iso*	2.59	2.58	1.80	2.62	0.27	0.24	0.36	0.20
15:0 *anteiso*	4.40	4.07	3.92	5.13	0.53	0.35	0.33	0.09
16:0 *iso*	1.25	1.09	0.66	0.70	0.12	0.04	0.55	0.40
17:0 *iso*	0.420	0.346	0.270	0.364	0.029	0.11	0.80	0.07
17:0 *anteiso*	0.512	0.487	0.339	0.465	0.090	0.19	0.42	0.26
18:0 *iso*	0.100	0.0492	0.114	0.0615	0.012	0.42	0.06	0.96

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil).

[2] *P* values for main effect means for diet (HF vs LF), CO, Diet × CO interactions. Only the branched-chain VFA (BCVFA) treatments were dosed with ^{13}C. The model also included a covariate (BCVFA mmol/d production) for the differences between diet for RDP and feed AA profile.

[3] The ^{13}C dose was provided as BCVFA (5 mg/d of ^{13}C from each isobutyrate, 2-methylbutyrate, and isovalerate). Percentage of recovered dose was calculated by 100 × (mg ^{13}C recovered in lipid C flow/ mg ^{13}C recovered in bacterial total C flow)

Table E.5. Dose recovery in bacterial aldehydes (ALD) in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation.

Total ^{13}C recovery in bacterial ALD C flow, µg/mg of ^{13}C dosed[3]	HF[1]		LF		SE	Significance[2]		
	– CO	+ CO	– CO	+ CO		Diet	CO	Diet × CO
11:0 *iso*	0.0192	0.0159	0.0126	0.0154	0.0041	0.24	0.92	0.22
11:0 *anteiso*	0.0060	0.0068	0.0050	0.0052	0.0013	0.26	0.49	0.51
12:0 *iso*	0.256	0.268	0.228	0.210	0.046	0.23	0.92	0.53
13:0 *iso*	0.202	0.235	0.165	0.191	0.040	0.34	0.38	0.91
13:0 *anteiso*	0.471	0.564	0.431	0.489	0.089	0.29	0.29	0.62
14:0 *iso*	1.06	0.83	0.70	0.73	0.12	0.16	0.36	0.29
15:0 *iso*	0.583	0.551	0.355	0.478	0.078	0.14	0.48	0.28
15:0 *anteiso*	1.44	1.32	0.97	1.21	0.14	0.12	0.55	0.19
16:0 *iso*	0.244	0.189	0.079	0.077	0.017	0.02	0.19	0.20
17:0 *iso*	0.0320	0.0323	0.0268	0.0295	0.0062	0.58	0.78	0.82
17:0 *anteiso*	0.0610	0.0296	0.0113	0.0102	0.0122	0.12	0.29	0.31
18:0 *iso*	0.0210	0.0172	0.0161	0.0120	0.0027	0.17	0.43	0.40
Other *iso* even ALD[4]	0.296	0.307	0.205	0.180	0.050	0.14	0.85	0.46
Other *iso* odd ALD[5]	0.264	0.239	0.166	0.218	0.043	0.11	0.61	0.16
Other *anteiso* ALD[6]	0.373	0.440	0.305	0.350	0.070	0.28	0.39	0.74

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil).

[2] *P* values for main effect means for diet (HF vs LF), CO, Diet × CO interactions. Only the branched-chain VFA (BCVFA) treatments were dosed with ^{13}C. The model also included a covariate (BCVFA mmol/d production) for the differences between diet for RDP and feed AA profile.

[3] The ^{13}C dose was provided as BCVFA (5 mg/d of ^{13}C from each isobutyrate, 2-methylbutyrate, and isovalerate).

[4] The sum of other *iso* even-chain BCALD, which were ALD with enrichment that were confirmed with individually dosed ^{13}C labeled Val and isobutyrate.

[5] The sum of other *iso* odd-chain BCALD, which were ALD with enrichment that were confirmed with individually dosed ^{13}C labeled Leu and isovalerate.

[6] The sum of other *anteiso* odd-chain BCALD, which were ALD with enrichment that were confirmed with individually dosed ^{13}C labeled Ile and 2-methylbutyrate.

Table E.6. Recovered dose distribution in bacterial aldehydes (ALD) in continuous cultures that were administered with either high or low forage diets that varied in corn oil supplementation.

Distribution of ^{13}C in bacterial lipid outflow, %[3]	HF[1]		LF		SE	Significance[2]		
	– CO	+ CO	– CO	+ CO		Diet	CO	Diet × CO
11:0 *iso*	0.0125	0.0112	0.0123	0.0159	0.0035	0.18	0.42	0.16
11:0 *anteiso*	0.00378	0.00475	0.00506	0.00544	0.00104	0.39	0.53	0.74
12:0 *iso*	0.160	0.176	0.220	0.230	0.046	0.14	0.66	0.92
13:0 *iso*	0.130	0.174	0.160	0.192	0.032	0.37	0.22	0.80
13:0 *anteiso*	0.293	0.377	0.469	0.521	0.083	0.08	0.30	0.78
14:0 *iso*	0.674	0.546	0.686	0.794	0.136	0.24	0.94	0.27
15:0 *iso*	0.374	0.401	0.367	0.493	0.072	0.56	0.34	0.50
15:0 *anteiso*	0.901	0.880	1.092	1.308	0.166	0.14	0.54	0.47
16:0 *iso*	0.155	0.130	0.083	0.086	0.016	0.03	0.53	0.33
17:0 *iso*	0.0198	0.0216	0.0360	0.0314	0.0048	0.19	0.78	0.57
17:0 *anteiso*	0.0401	0.0189	0.0082	0.0132	0.0093	0.08	0.53	0.15
18:0 *iso*	0.0134	0.0121	0.0161	0.0136	0.0022	0.13	0.53	0.40
Other *iso* even ALD[4]	0.185	0.205	0.214	0.196	0.045	0.73	0.98	0.55
Other *iso* odd ALD[5]	0.171	0.173	0.159	0.220	0.036	0.33	0.15	0.17
Other *anteiso* ALD[6]	0.230	0.289	0.360	0.376	0.070	0.21	0.49	0.66

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil).

[2] *P* values for main effect means for diet (HF vs LF), CO, Diet × CO interactions. Only the branched-chain VFA (BCVFA) treatments were dosed with ^{13}C. The model also included a covariate (BCVFA mmol/d production) for the differences between diet for RDP and feed AA profile.

[3] The ^{13}C dose was provided as BCVFA (5 mg/d of ^{13}C from each isobutyrate, 2-methylbutyrate, and isovalerate). Percentage of recovered dose was calculated by 100 × (mg ^{13}C recovered in lipid C flow/ mg ^{13}C recovered in bacterial total C flow)

[4] The sum of other *iso* even-chain BCALD, which were ALD with enrichment that were confirmed with individually dosed ^{13}C labeled Val and isobutyrate.

[5] The sum of other *iso* odd-chain BCALD, which were ALD with enrichment that were confirmed with individually dosed ^{13}C labeled Leu and isovalerate.

[6] The sum of other *anteiso* odd-chain BCALD, which were ALD with enrichment that were confirmed with individually dosed ^{13}C labeled Ile and 2-methylbutyrate.

Appendix F. Completed amino acid flow and profile tables from dual flow continuous cultures

Table F.1. Bacterial AA flows in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation.

Item	HF[1]				LF				SEM	Significance[2]			
	– CO		+ CO		– CO		+ CO						
	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA	Diet × CO × BCVFA
AA Flow g/d													
Cys[3]	0.069	0.085	0.078	0.074	0.072	0.078	0.072	0.067	0.005	0.22	0.35	0.30	0.46
Met[3]	0.135	0.170	0.166	0.151	0.150	0.177	0.163	0.154	0.009	0.41	0.94	0.16	0.62
Asp	0.77	0.89	0.80	0.79	0.86	0.91	0.79	0.89	0.04	0.04	0.10	0.02	0.10
Thr	0.351	0.408	0.369	0.365	0.403	0.418	0.361	0.407	0.020	0.07	0.14	0.04	0.09
Ser	0.306	0.356	0.320	0.313	0.351	0.370	0.319	0.356	0.017	0.03	0.10	0.04	0.11
Glu	0.810	0.954	0.843	0.831	0.915	0.970	0.840	0.947	0.043	0.05	0.10	0.02	0.07
Pro	0.227	0.273	0.242	0.237	0.268	0.265	0.230	0.256	0.014	0.31	0.10	0.11	0.05
Gly	0.341	0.406	0.356	0.352	0.386	0.405	0.355	0.394	0.017	0.06	0.08	0.01	0.06
Ala	0.437	0.518	0.453	0.446	0.495	0.518	0.455	0.506	0.020	0.03	0.05	0.01	0.04
Val	0.418	0.477	0.426	0.435	0.470	0.515	0.429	0.501	0.021	0.01	0.11	< 0.01	0.17
Ile	0.371	0.429	0.380	0.387	0.418	0.455	0.382	0.445	0.019	0.01	0.08	< 0.01	0.09
Leu	0.483	0.570	0.497	0.495	0.549	0.604	0.505	0.583	0.027	0.01	0.08	< 0.01	0.11
Tyr	0.308	0.358	0.316	0.314	0.345	0.367	0.317	0.355	0.017	0.05	0.08	0.02	0.11
Phe	0.304	0.358	0.318	0.314	0.345	0.368	0.316	0.355	0.015	0.02	0.06	0.01	0.05
His	0.106	0.123	0.110	0.110	0.120	0.129	0.110	0.124	0.006	0.01	0.07	0.01	0.10
Lys	0.468	0.551	0.490	0.483	0.546	0.575	0.508	0.568	0.023	< 0.01	0.12	0.01	0.05
Arg[4]	0.312	0.321	0.321	0.283	0.314	0.352	0.305	0.362	0.017	0.04	0.52	0.14	0.14
Trp	0.100	0.118	0.107	0.105	0.110	0.116	0.106	0.112	0.006	0.25	0.22	0.02	0.08

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] P values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] Interaction of CO and BCVFA supplementation ($P \leq 0.02$).

[5] Interaction of Diet and BCVFA supplementation ($P = 0.01$).

Table F.2. Effluent AA flows in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation.

	HF[1]				LF				SEM	Significance[2]		
	– CO		+ CO		– CO		+ CO					
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA
AA, % DM	12.8	14.9	12.5	14.3	12.6	14.1	12.4	13.2	0.9	0.12	0.14	< 0.01
Flow, g/d												
Total AA	11.7	13.5	11.8	13.6	11.4	13.3	11.4	11.9	0.9	0.07	0.35	< 0.01
Total BCAA[3]	2.49	2.86	2.51	2.91	2.46	2.82	2.46	2.62	0.16	0.15	0.63	< 0.01
Cys[4]	0.121	0.172	0.131	0.158	0.108	0.132	0.134	0.102	0.017	0.03	0.85	0.14
Met[5, 6]	0.208	0.258	0.238	0.242	0.196	0.232	0.203	0.193	0.016	< 0.01	0.50	0.01
Asp	1.23	1.40	1.26	1.40	1.20	1.38	1.21	1.26	0.11	0.12	0.60	< 0.01
Thr	0.588	0.659	0.589	0.653	0.571	0.660	0.571	0.586	0.051	0.14	0.24	< 0.01
Ser	0.603	0.660	0.585	0.649	0.581	0.671	0.562	0.591	0.050	0.18	0.07	< 0.01
Glu	1.55	1.82	1.57	1.80	1.59	1.86	1.58	1.68	0.15	0.91	0.33	< 0.01
Pro	0.582	0.674	0.597	0.705	0.553	0.692	0.556	0.605	0.061	0.12	0.67	< 0.01
Gly	0.674	0.780	0.665	0.773	0.657	0.753	0.637	0.665	0.055	0.02	0.11	< 0.01
Ala	0.794	0.937	0.785	1.054	0.790	1.032	0.782	0.787	0.114	0.44	0.52	0.01
Val	0.713	0.840	0.712	0.856	0.718	0.832	0.713	0.729	0.064	0.15	0.30	< 0.01
Ile	0.627	0.733	0.654	0.750	0.624	0.730	0.639	0.673	0.048	0.22	> 0.99	< 0.01
Leu	1.15	1.28	1.15	1.30	1.12	1.26	1.11	1.22	0.06	0.21	0.77	< 0.01
Tyr	0.458	0.544	0.469	0.549	0.470	0.539	0.479	0.502	0.040	0.62	0.83	< 0.01
Phe	0.591	0.697	0.591	0.693	0.551	0.650	0.558	0.585	0.056	< 0.01	0.34	< 0.01
His	0.237	0.268	0.231	0.264	0.231	0.266	0.227	0.234	0.015	0.11	0.08	< 0.01
Lys	0.726	0.808	0.716	0.808	0.683	0.812	0.700	0.730	0.064	0.22	0.49	0.01
Arg[6]	0.596	0.708	0.626	0.657	0.542	0.608	0.527	0.538	0.043	< 0.01	0.18	0.01
Trp	0.218	0.234	0.230	0.240	0.195	0.199	0.183	0.196	0.012	< 0.01	0.91	0.11

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] P values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] Branched-chain AA (BCAA) is the sum of Val, Ile, and Leu.

[4] Interaction of Diet and BCVFA supplementation ($P = 0.07$).

[5] Interaction of Diet and CO supplementation ($P \leq 0.09$).

[6] Interaction of CO and BCVFA supplementation ($0.01 \leq P \leq 0.09$).

Table F.3. Non-bacterial AA profile in continuous cultures that were administered with either high or low forage diets that varied in corn oil and branched-chain VFA supplementation.

	HF[1]				LF				SEM	Significance[2]		
	– CO		+ CO		– CO		+ CO					
Item	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA	– BCVFA	+ BCVFA		Diet	CO	BCVFA
Bacterial AA Profile, g/100 g total AA												
Cys	1.09	1.42	1.28	1.22	0.85	0.78	1.28	0.88	0.32	0.18	0.54	0.82
Met	1.45	1.40	1.47	1.34	1.09	0.93	0.82	0.85	0.28	< 0.01	0.51	0.61
Asp	8.5	8.3	8.1	8.6	7.8	8.0	8.6	7.9	0.6	0.37	0.70	0.82
Thr[3]	4.40	4.11	3.90	4.07	3.86	4.22	4.35	3.81	0.29	0.76	0.53	0.67
Ser	5.53	4.96	4.91	4.76	5.37	5.33	5.05	5.15	0.17	0.12	0.01	0.16
Glu	13.7	14.1	13.6	13.7	15.9	15.7	15.5	16.5	0.4	< 0.01	0.85	0.18
Pro[4]	6.68	6.58	6.79	6.54	6.74	7.57	6.81	7.96	0.29	< 0.01	0.39	0.02
Gly	6.20	6.13	5.91	5.97	6.38	6.15	5.88	6.11	0.13	0.35	0.01	0.99
Ala[3]	6.66	6.88	6.36	8.05	6.97	8.19	6.80	6.34	0.68	0.84	0.53	0.15
Val[4]	5.38	5.96	5.55	5.90	5.82	5.35	5.89	5.02	0.28	0.26	0.80	0.52
Ile	4.78	5.02	5.40	5.21	4.81	4.87	5.34	4.97	0.35	0.45	0.02	0.62
Leu[3, 4]	12.65	11.97	13.67	11.95	13.44	12.37	12.72	14.49	1.22	0.05	0.09	0.23
Tyr[4]	2.78	3.02	2.72	3.26	2.91	2.94	3.37	3.08	0.21	0.38	0.19	0.37
Phe	5.29	5.52	5.13	5.33	4.89	4.93	4.99	5.00	0.23	0.01	0.72	0.31
His	2.47	2.39	2.42	2.25	2.57	2.52	2.43	2.54	0.13	0.01	0.11	0.31
Lys[3]	4.79	4.05	3.96	4.57	3.04	3.97	4.00	3.19	0.51	0.04	0.93	0.99
Arg	5.33	6.34	6.31	5.34	5.51	4.72	4.59	4.29	0.74	0.03	0.44	0.55
Trp[3]	2.30	1.89	2.52	1.96	2.03	1.51	1.60	2.00	0.33	0.01	0.49	0.05

[1] Treatments are high forage (HF, 67% forage), low forage (LF, 33% forage), – CO (no additional supplemented fat), + CO (3% DM supplemented fat as corn oil), – BCVFA (no supplemented branched-chain volatile fatty acids), + BCVFA (2.15 mmol/d of each isobutyrate, 2-methylbutyrate, and isovalerate)

[2] P values for main effect means for diet (HF vs LF), CO, BCVFA or interactions; interactions not shown have $P > 0.10$.

[3] Interaction of Diet, CO, and BCVFA supplementation ($0.01 \leq P \leq 0.10$).

[4] Interaction of Diet and BCVFA supplementation ($0.01 \leq P \leq 0.09$).