Improving the Understanding of Factors Driving Rumen Fermentation

Claire Gleason

Dissertation submitted to the faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Animal and Poultry Sciences

Robin R. White, Advisor
Thomas Bain Wilson
Gonzalo Ferreira
D. Michael Denbow

April 28, 2021
Blacksburg, Virginia

Keywords: volatile fatty acids, nutrient supply, rumen microbiome, rumen epithelium

Improving the Understanding of Factors Driving Rumen Fermentation

Claire Gleason

Abstract

Ruminant livestock maintain an important role in meeting the nutrient requirements of the global population through their unique ability to convert plant fiber into human-edible meat and milk products. Volatile fatty acids (**VFA**) produced by rumen microbial fermentation of feed substrates represent around 70% of the ruminant animal's metabolic energy supply. Rumen fermentation profiles may directly impact productivity because the types of VFA produced are utilized at differing efficiencies by the animal. Improving our understanding of factors that control these fermentative outcomes would therefore aid in optimizing the productive efficiency of ruminant livestock. Improvements in animal efficiency are now more important than ever as the livestock industry must adapt to continue meeting the nutritional needs of a growing global population in the context of increased resource restrictions and requirements to lower the environmental impact of production. The relationship between diet and VFA ultimately supplied to the animal is complex and poorly understood due to the influence of numerous nutritional, biochemical, and microbial variables. The central aim of this body of work was therefore to explore and characterize how fermentation dynamics, rumen environmental characteristics, and the rumen microbiome behave in response to variations in the supply of fermentative substrate. The objective of our first experiment was to describe a novel *in vitro* laboratory technique to rank livestock feeds based on their starch degradability. This experiment also compared the starch degradation rates estimated by the *in vitro* method to the rates estimated by a traditional *in situ* method using sheep. A relationship between the degradation rates determined by these two procedures was observed, but only when feed nutrient content was accounted for. While this *in*

vitro approach may not be able to reflect actual ruminal starch degradation rates, it holds potential as a useful laboratory technique for assessing relative differences in starch degradability between various feeds. Our second experiment aimed to measure changes in VFA dynamics, rumen environmental characteristics, and rumen epithelial gene expression levels in response to dietary sources of fiber and protein designed to differ in their rumen availabilities. Conducted in sheep, this study utilized beet pulp and timothy hay as the more and less available fiber source treatments, respectively, and soybean meal and heat-treated soybean meal as the more and less available protein source treatments, respectively. Results indicated that rumen environmental parameters and epithelial gene expression levels were not significantly altered by treatment. However, numerous shifts in response to both protein and fiber treatments were observed in fermentation dynamics, especially in interconversions of VFA. The objective of the third investigation was to assess whether the rumen microbiome can serve as an accurate predictor of beef and dairy cattle performance measurements and compare its predictive ability to that of diet explanatory variables. The available literature was assembled into a meta-analysis and models predicting dry matter intake, feed efficiency, average daily gain, and milk yield were derived using microbial and diet explanatory variables. Comparison of model quality revealed that the microbiome-based predictions may have comparable accuracy to diet-based predictions and that microbial variables may be used in combination with diet to improve predictions. In our fourth experiment, the objective was to investigate rumen microbial responses to the fiber and protein diet treatments detailed in Experiment 2. Responses of interest included relative abundances of bacterial populations at three taxonomic levels (phylum, family, and genus) in addition to estimations of community richness and diversity. Numerous population shifts were observed in response to fiber treatment. Prominent fibrolytic population abundances as well as richness and

diversity estimations were found to be greater with timothy hay treatment and lower with beet pulp whereas pectin degraders increased in abundance on beet pulp. Microbial responses associated with protein treatment were not as numerous but appeared to reflect taxa with roles in protein metabolism. These four investigations revealed that significant changes can occur in VFA fermentation and rumen microbial populations when sources of nutrient substrates provided in a ruminant animal's diet are altered and that a new approach may be useful in investigating degradation of another important substrate for fermentation (starch) in a laboratory setting. Our findings also determined that animal performance can be predicted to a certain extent by rumen microbial characteristics. Collectively, these investigations offer an improved understanding of factors that influence the process of converting feed to energy sources in the ruminant animal.

Improving the Understanding of Factors Driving Rumen Fermentation

Claire Gleason

General Audience Abstract

Ruminant animals, such as beef cattle, dairy cattle, and sheep, play a major role in delivering essential nutrients to the human population through their provision of meat and dairy products. The current growth projections of the global population, in addition to increased concerns surrounding greenhouse gas emissions and restrictions on resources such as land and water make it important for us to consider ways of optimizing the productivity of these animals. A unique feature of ruminants is their ability to conduct microbial fermentation of large amounts of plant matter in their rumens to produce energetically valuable compounds called volatile fatty acids (**VFA**), which are the primary source of energy that the animals use for growth, reproduction, and milk production. One promising way of improving animal productivity is to increase the amount of energy from the diet that becomes available to fuel the animal's body processes; however, the process of converting feed to VFA is complicated and currently not well understood. The overall aim of this body of work was therefore to explore various nutritional, ruminal, and microbial factors that are known to impact fermentation in order to 1) increase our understanding of how these factors interconnect and 2) put us in a better position to manipulate these factors for optimal animal performance. The goal of our first experiment was to devise and use a novel laboratory technique to rank livestock feeds based on the degradability of their starch content, which is an important substrate for VFA fermentation. Our observations indicate that this technique may be a useful tool to help us determine relative differences between feeds based on their starch degradabilities in a laboratory setting. Our second experiment investigated the effects of feeding varying sources of fiber (beet pulp and timothy hay) and protein (heat-treated

and untreated soybean meals) to sheep in terms of their VFA fermentation, rumen conditions, and the expression of certain key genes in the epithelial tissue of the rumen wall. While rumen environmental characteristics and epithelial gene expression remained largely unchanged, numerous key aspects of VFA fermentation, predominantly carbon exchanges between different VFA, were altered in response to nutrient source. The third investigation described in this work examined the ability of the microbial populations responsible for rumen fermentation to explain variation in beef and dairy cow productivity compared with the ability of diet characteristics to explain this variation. Using statistical methods to analyze the reports currently available in scientific literature, our findings indicate that the rumen microbiome and diet may exert independent effects on productivity levels and that the microbiome may be used to enhance diet-based predictions of animal performance. Finally, we explored variations in the sheep rumen microbiome in response to the diet treatments utilized in Experiment 2. We observed minimal impact of protein source on the microbiome, but numerous microbial responses were evident when fiber source was varied. These responses included decreases of fiber-degrading bacterial populations and increases in pectin-degrading populations when beet pulp was fed compared to timothy hay. Taken together, these experiments help to provide us with a more comprehensive picture of the numerous factors involved in the process of converting feed to a usable form of energy for ruminant livestock.

Acknowledgements

I would like to thank my parents, Terry and Susan Gleason, for their incredible love and support on this journey. Thank you for all the investments you've made in my education and all the opportunities that you've given me that have helped me reach this point in my life.

A huge thanks goes to my advisor, Dr. Robin White. Thank you for taking me onboard and for your mentorship these past four years. You've made me a better researcher, thinker, and human being. It has been an honor and a privilege to be a part of your lab. Thank you also to my committee members: Dr. Mike Denbow, Dr. Gonzalo Ferreira, and Dr. Bain Wilson. Your guidance, support, and encouragement have been invaluable.

Finally, I would like to thank a large number of people who have been my co-authors, lab mates, or who have provided assistance during my research trials: Dr. Hollie Schramm, Dr. Robert Settlage, Dr. Andrea Bedford, Dr. Doug Liebe, Dr. Chuck Zumbaugh, Linda Beckett, Ty Davis, Tanner Price, Barbara dos Reis, Sathya Sujani, Emily Conner, Nicholas Dias, Keri Hardin, Adam Murray, Kaitlyn Bahrs, Melissa Barnes, Avery Byrd, Natalie Cockram, Anna Foley, Emery Hawkins, Tara Ingersoll, Emily Llaneras, Luke Moore, Maiya Pullin, Baker White, and Jennifer Yang.

TABLE OF CONTENTS

CHAPTER 1: A Role for Beef Cattle in Sustainable U.S. Food Production

This chapter is published as: Gleason, C. B., and R. R. White, 2019. BEEF SPECIES-RUMINANT NUTRITION CACTUS BEEF SYMPOSIUM: A role for beef cattle in sustainable US food production. Journal of Animal Science, 97:4010-4020.

ABSTRACT

The increasing global population, limited resource availability, and global focus on reducing greenhouse gas (**GHG**) emissions put pressure on animal agriculture industries to critically evaluate and optimize the role they play in a sustainable food production system. The objective of this review is to summarize evidence of the various roles that the U.S. beef industry plays in the U.S. and global agricultural systems. As the world's largest beef producer, the U.S. reaps considerable economic benefit from the beef industry through strong domestic and international demand, as well as employment opportunities for many Americans. Beef production contributes to GHG emissions, land use, and water use, among other critical environmental impacts but provides an important source of essential micronutrients for human consumption. The U.S. beef industry provides sufficient product to meet the protein, vitamin B12, omega-3 and -6 fatty acid requirements of 43, 137, 47, and 487 million people, respectively. In the U.S., beef production was estimated to account for 53% of GHG emissions from U.S. animal agriculture and 25% of GHG emissions from all of U.S. agriculture. Footprinting studies suggest that much of the land use and water use associated with beef production are attributed to the development of feed crops or pastureland. On a global scale, beef from U.S. origin is exported to numerous developed and developing countries, representing an important international nutrient routing. Along with other prominent beef-producing nations, the United States continues to pursue a greater level of sustainability in its cattle industry, which will bear important implications for future global food security. Efforts to reduce the environmental impacts of beef production will likely be the strongest drivers of enhanced sustainability.

Keywords: beef industry, environmental impact, human nutrition, sustainability

INTRODUCTION

The global population is expected to exceed 9.8 billion by the year 2050 (UN DESA, 2017). Along this same timescale, improved affluence in developing nations is expected to increase global demand for meat and milk (Seale, 1998; Delgado, 2003). Globally, land and water availability are already limited (Gomiero et al., 2011; Hertel, 2011). As a result, concern exists about the opportunity to rely on food production, as it currently functions, to meet this demand increase. Agricultural food production also contributes to atmospheric concentrations of greenhouse gases (**GHGs**) like methane (CH_4), nitrous oxide (N_2O) and carbon dioxide (CO_2) (United States Environmental Protection Agency (**EPA**), 2018). The current challenge requires food production to improve land and water use efficiency and decrease GHG emissions while developing adaptive capacity in the face of climate variability (Nardone et al., 2010). A commonly proposed solution to these challenges is to improve "sustainability" of food production systems (Gomiero et al., 2011). Sustainability is defined as a balance between social acceptability, environmental responsibility, and economic viability (Brundtland, 1991; Committee on Incorporating Sustainability in the U.S. Environmental Protection Agency et al., 2011; Committee on Sustainability Linkages in the Federal Government et al., 2013). By improving sustainability, ideally, the environmental impact of a system can be reduced so that societal demands for food can be met within the bounds of resource availability and climate conditions.

CONTRIBUTIONS OF BEEF TO U.S. AGRICULTURE

Beef production in the United States is a socioeconomically valuable system (USDA/NASS, 2018). The U.S. is not only the largest net consumer of beef globally, but it also

houses the largest fed cattle industry in the world (OECD and FAO, 2014). The beef production system is comprised of three sectors: the cow-calf, stocker/backgrounder, and feedlot. Cow-calf operations breed cows and bulls and sell weaned calves. Stocker operations purchase weaned calves, feed them on pasture, and sell yearlings. Backgrounding operations feed weaned calves forage-based rations out of feed bunks in preparation for entry into a feedlot. Feedlots purchase weaned calves from cow-calf operations, cull dairy calves or yearlings from stocker/backgrounder operations, and market finished animals for harvest. Studies have cataloged the environmental impact of each sector (Beauchemin et al., 2010) and comparisons among sectors can be useful in highlighting opportunities to improve the overall role beef production plays in the agricultural environmental footprint.

Sustainability, however, must consider more than just environmental impact. Sustainability balances economic, environmental, and social concerns; therefore, assessments of sustainability should encompass metrics for each of these areas of focus. This review characterizes the human nutritional, environmental, and economic roles that the beef industry plays in the U.S. and global agricultural systems with an emphasis on how the industry might consider continual adaptation to optimize those roles.

Nutritional Contributions

Contributions of beef cattle to the supply of human-edible nutrients provided by U.S. agriculture are summarized in Table 1 and were derived from the data presented previously (White and Hall, 2017). Along with other animal products, beef meets the definition of a "complete protein" because it contains all 9 amino acids that must be obtained via the human diet. Very few plants provide all essential amino acids. Beef is therefore considered a higher

quality protein source than the vast majority of plant-based protein foods. Surprisingly, beef provides only 5% of the protein available for consumption in the United States today (Table 1). Amino acids from beef production range between 3.7 and 7% of the total domestic supplies, with Lys, Met and His being in the greatest supplies (Table 1). Lys is indispensable for its role as a building block for the synthesis of proteins such as collagen. Additionally, it supports the process of beta-oxidation as a precursor to carnitine (Tanphaichitr and Broquist, 1973). Cereal grains possess low quantities of Lys, frequently making Lys the limiting amino acid in human and animal diets. As a precursor to succinyl-CoA and a host of other biologically relevant molecules, Met is another essential amino acid. It is involved in activities ranging from fat metabolism to disease prevention (Martínez et al., 2017). Histidine was once thought to be only essential for children but is now considered essential in all life stages because it is the precursor to the neurotransmitter histamine, which is involved in appetite regulation, fat mobilization, and metabolic rate (DiNicolantonio et al., 2018).

Besides amino acids, beef also provides notable quantities of vitamin B3, vitamin B6, and vitamin B12. Vitamin B3, or niacin, serves as a precursor to co-enzymes vital to metabolism (Surjana et al., 2010). Vitamin B6 (pyridoxine) is important for proper immune, nervous, and endocrine function. Deficiencies of B6 have been linked to neurological disorders and birth defects (Ahmad et al., 2013). Also known as cobalamin, vitamin B12 is essential for proper metabolic function as well as DNA synthesis and modification (Green et al., 2017). Interestingly, vitamin B12 is only synthesized by microorganisms, making it an essential nutrient for humans to absorb from the diet. Naturally synthesized vitamin B12 can only be obtained through the consumption of animal-origin foods. Beef and other ruminant animal products are especially valuable sources of B12 owing to the large quantities of microbes inhabiting the ruminant

gastrointestinal tract that are capable of cobalamin synthesis (Gille and Schmid, 2015). Following synthesis, cobalamin storage takes place in the animal's muscle and liver tissue (Martens et al., 2002). Although severe cases are uncommon in developed countries, vitamin B12 deficiencies are most often noted in individuals who restrict or eliminate the consumption of animal products, or who lack proper access to these foods (Pawlak et al., 2013; Green et al., 2017). Well-established manifestations of deficiency include neurologic and psychiatric disorders, as well as anemia (Bar-Shai et al., 2011).

Beef is a rich source of dietary minerals, providing 2.7% of the iron, 2.5% of the selenium, and 6.6% of the zinc available for human consumption in the United States (Table 1). While significant quantities of minerals may be found in plant-based foods, they are typically less biologically available than those present in animal products. Heme iron in meat, for instance, is more readily absorbed than the non-heme iron present in plants. As myoglobin is digested, the heme iron released is maintained in an optimal state for absorption by the nearby products of globin degradation. Iron deficiency is therefore less common in populations that consume adequate amounts of meat or offal (Uzel and Conrad, 1998). According to Solomons (2001), poor zinc uptake has been noted in both whole grain- and soy-based diets. The impaired bioavailability of zinc in plants is attributed to anti-nutritional factors such as tannins and phytic acid. Beef and other animal meats are devoid of such compounds and just 100 g of beef contain approximately 45.3% of an adult male's daily zinc requirement (Solomons, 2001). A key micronutrient in proper immune and reproductive function, selenium from beef has also been reported as highly available to humans (Van Der Torre et al., 1991). According to Holden et al. (1991), beef is "the most abundant single food source of selenium in the North American diet."

Broccoli is also rich in selenium, but the complex food matrix in which the mineral is bound may significantly lower its bioavailability when consumed (Thiry et al., 2012).

Beef has often been criticized as having detrimental effects on the heart due its saturated fatty acid (**SFA**) content. However, this claim has recently come under scrutiny and a considerable amount of research has been unable to link SFA intake to increased risk of cardiovascular disease (Lawrence, 2013). Indeed, many years' worth of recommendations to reduce red meat consumption have not led to improved health in the U.S. population. Beef instead remains a significant source of heart healthy *cis*-monounsaturated fatty acids and the long-chain omega-3 fatty acids eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) (Vahmani et al., 2015). As identified in White and Hall (2017), beef contributes to U.S. food supplies by providing over 10% of the EPA+DHA and 7% of the arachidonic acid (**AA**) available in the nation. Furthermore, beef provides fatty acid biohydrogenation products such as rumenic and vaccenic acids. These fatty acids have been identified as possessing hypolipidemic and anticarcinogenic properties (Vahmani et al., 2015).

When considering the role of beef in providing a high-quality nutrient supply to the United States, the provision of these critical nutrients is an important aspect. Continuing to fine-tune management to optimize product quality and fatty acid composition may be an important area of work to reinforce this nutritional contribution in the future.

Economic Contributions

The United States is currently the largest beef producing country in the world. Between 2014 and 2018, the United States was responsible for nearly 20% of global beef production, averaging approximately 12.0 million metric tons in carcass weight equivalent annually

(USDA/ERS, 2018a; USDA/FAS, 2018a). Domestic beef consumption in the United States is also larger than consumption in any other country, averaging 12.1 billion kg in total weight annually between 2002 and 2015 (USDA/ERS, 2018b). Unsurprisingly, beef production is one of the nation's most important industries, generating $67.4 billion in direct cash receipts in 2017 /ERS, 2018c).

The United States is currently the world's fourth largest exporter of beef, totaling over 1.26 million metric tons of product in 2017 with a dollar value of $7.3 billion (USMEF, 2017; USDA/FAS, 2018a). The Nation is also the top global importer, with over 1.35 million metric tons of foreign beef imported in 2017 (USDA/FAS, 2018a). Export value, however, exceeded import value by $1.6 billion due to the fact that the U.S. mainly imports inexpensive lean beef trimmings for hamburger rather than high-priced cuts (Speer, 2018). Leading importers of U.S. beef, including beef variety meats, are listed in Table 2. At the time of this writing, beef exports are following an upward trend in practically every market (USMEF, 2018). Key markets including Canada, South Korea, and Japan are forecasted to import more U.S. beef due to a combination of competitive prices, abundant supply, and decreased Australian exports (USDA/FAS, 2018a). Strong foreign demand has led to record-breaking volume and value of exports with Asian markets being the major drivers of this increase. Increased affluence and a growing middle class in many Pacific nations have helped fuel the demand for American beef and greater market access has allowed the United States to profit from this demand. The recent reopening of the Chinese market to U.S. beef in June of 2017 yielded an export of 3,655 metric tons valued at $33 million during the first half of 2018. Closed to U.S. beef for 13 yr, China has become the world's second largest beef importer, growing from $275 million in imports during 2012 to $2.5 billion in 2016 (USDA/FAS, 2017a; USDA/FAS, 2018a). Growing demand for

American beef has also been noted in the Indonesian market. Increased ease of export to Indonesia as well as advocacy efforts on the parts of the USDA Foreign Agricultural Service and the U.S. Meat Export Federation have resulted in a record of $53.7 million in exports to Indonesia in 2017, an increase of almost 40% from the previous year (USDA/FAS, 2018b). Because of the significant impact U.S. beef makes in foreign markets and food supplies, decisions to change aspects of our domestic beef production must be made with the awareness that foreign consumers may also be impacted.

The animal agriculture industry in the United States employs over 1 million people between animal food manufacturing, animal production, and animal processing (USDL/BLS, 2014). Due to its significance for the U.S. economy, beef production takes a prominent position in these sectors. The USDA National Agricultural Statistics Service's most recent agricultural census reported 727,906 beef cattle farms (including ranches and research farms) in operation (USDA/NASS, 2012). Raising beef cattle therefore provides a livelihood for a significant number of Americans. The cattle feeding industry also employs numerous professionals, which cumulatively reflect a substantial economic impact on the U.S. economy.

At present, the major economic roles of beef cattle include generating export income and providing jobs for Americans. As the industry continues to develop, focus should be put on developing high-quality jobs. Continued integration of technology into production is critical for this generation of high-quality jobs but will require shifts in the workforce development strategy and continuing workforce education. Optimizing the role of the U.S. beef industry as an exporter has been a focus for decades and the success of this effort is demonstrable in the statistics on income from export. As the industry evolves, continuing to consider the benefits of this export-driven economy in the context of collateral social and environmental concerns is essential.

Environmental Contributions

Beef cattle account for 52% of emissions from animal agriculture and 25% of all agricultural emissions in the U.S. (White and Hall, 2017; Mitloehner, 2018). Emissions attributed to beef cattle production include CO_2 emissions from synthesis of commercial fertilizer, herbicide, seed, and other cropping-system inputs; on-farm CO_2 emissions from land management and transportation; CH_4 emissions from enteric fermentation and manure storage; direct and indirect N_2O emissions from manure storage; CO_2 emissions from infrastructure upkeep; and other sources. Beef cattle remain the predominant contributors of CH_4 emissions and were responsible for 71% of total enteric CH_4 emissions from livestock in 2016 (EPA, 2018). System boundaries for establishing a carbon footprint for beef production typically extend from the inputs to the cropping system through the feedlot or slaughterhouse gate. For most U.S. beef production enterprises, the cow-calf operation contributes the greatest to the whole-system emission primarily because of enteric fermentation from the cow herd (Beauchemin et al., 2010; Asem-Hiablie et al., 2019).

Water use estimates from beef cattle production vary considerably based on accounting approach and production system and are typically estimated between 200 L/kg and 4,000 L/kg depending on the different sources of water included (Beckett and Oltjen, 1993; Oltjen and Beckett, 1996; White et al., 2014). Some estimates of beef production water use are over 10,000 L/kg when accounting for all sources of water (Mekonnen and Hoekstra, 2012). Water use is broadly classified into 3 categories: blue water includes surface and ground water; green water refers to rainwater; and grey water refers to the volume of freshwater required for diluting pollutants to adequate water quality standards (Mekonnen and Hoekstra, 2012). Sources of direct water use on beef operations typically include irrigation water; sanitation and processing water;

and animal drinking water. Irrespective of the water footprint estimate, most studies agree that land-applied water is the greatest contributor to a C-footprint (Beckett and Oltjen, 1993; White and Capper, 2013). In 2013, 81.2 billion liters of water was applied to crops in the United States (USDA/NASS, 2013). Globally, only 10% to 30% of irrigation water is utilized by plants for growth (Wallace, 2000), suggesting that improving efficiency of irrigation water use within crop production would have notable impacts on the water footprint of beef. By comparison to the irrigation water usage for feed production, the 4 to 15 liters of water per kg feed intake consumed directly by livestock (Winchester and Morris, 1956; Mader and Davis, 2004), or 123 liters of daily drinking water per kg of beef (White et al., 2014), is a minimal contribution.

Land use associated with beef cattle production is considered both a resource use and a benefit associated with production. Biodiversity preservation on rangeland is important and the impacts of herbivory on healthy grasslands have clearly been demonstrated. Oltjen and Beckett (1996) noted that most assessments of land use in beef production incorrectly assume that all land used for beef production could also be used for human food production. Estimates of land use for beef production range from roughly 15 m^2/kg for intensive systems (Nguyen et al., 2010) to nearly 100 m^2/kg for grass-finishing systems (Capper, 2012). In most U.S. production systems, a substantial amount of that land footprint (e.g., roughly 66% in White and Capper (2013)) is associated with grazing. It is difficult to estimate the number of cattle housed on rangeland and pastureland that is not croppable in the United States, but it is expected to be the majority of beef cows. This assumption implies that the majority of improvements in beef land use could be accomplished by optimizing stocking density in grazing systems. A key requirement of stocking density optimization is improvement in the efficiency of forage use, which can be achieved through the employment of specific grazing systems such as rotational or

strip grazing. Strip grazing, for example, may result in upwards of 80% forage use efficiency when utilized (Stewart et al., 2012). Finding the best grazing management or forage optimization strategy is likely something that must be accomplished on the individual farm scale considering constraints like capital, labor, climate, and other factors.

BEEF CATTLE AS RECYCLING ENGINES

Use of Non-Human-Edible Resources

A critical aspect of beef cattle production that is unique among livestock systems in the United States is the extensive use of land area that could not otherwise be harvested for food production (Oltjen and Beckett, 1996). In particular, beef cattle are the primary species used to graze the 96 million acres of rangeland (USDA Forest Service, 2018) available in the United States. Forage accounts for approximately 80.8% of the feed needed to produce market-ready beef and the total amount of forage needed to produce 1 grain-finished feedlot animal is estimated at 6,411 kg on a dry matter (**DM**) basis (NRC, 2016). The amount of grain required is estimated at less than 10%, or 793.4 kg DM (NRC, 2016). The remaining 10% of the life-cycle diet is comprised of by-products of the various grain and pulse processing industries. Consumption of these human-inedible resources likely exceeds 800 kg DM per animal. Optimizing the conversion of human-inedible feed resources into human-edible food is crucial for solidifying the role of beef in a sustainable human diet.

For some time, beef production has been touted as a critical way to generate high-quality human-edible protein from human-inedible products. However, when the analysis by White and Hall (2017) is assessed specifically for beef product contributions, beef production in the United States only provides sufficient protein to meet the requirements of 14% of the population (Table

1). Other essential micronutrients like vitamin B12 and AA appear to be more important contributions of beef than protein and individual amino acids (Table 1). As such, when discussing the role of beef cattle in producing human-edible products from non-human-edible resources, there is likely a need to identify more important contributors toward these nutrient supplies.

As mentioned above, a critical cost of the ruminant animal's unique ability to synthesize human-edible nutrients from low-quality forage is the production of methane. A long history of methane research (Johnson and Johnson, 1995) and models of methane prediction (Mills et al., 2003; Ellis et al., 2010) all agree that forage-based diets generate higher methane production than grain-based diets. This suggests that converting cows from forage (range, pasture, or stored forage) diets to feedlot rations might be an efficient way to reduce methane emissions; however, the economic and social ramifications of this approach and the associated resource use make it simply infeasible. The USDA/NASS (2018) estimates there are roughly 32 million beef cows in the United States. On a pasture-based diet (13 kg/d DMI), the equation of Moe and Tyrell (1979) predicts a 567 kg beef cow to produce 371 g of CH_4 per day. If the 32 million cows in the United States each emit 371 g of CH_4 per day, their annual emissions equate to 4.3 million metric tons of CH_4. If instead, they were emitting closer to 180 g of CH_4 per day (as might be expected from a grain-based ration), their net annual emissions would equate to 2.1 million metric tons of CH_4. This reduction equates to over 50 million metric tons of CO_2 equivalents. Considering that total U.S. emissions in 2016 were estimated at 6.5 billion metric tons of CO_2 equivalents, a 50 million metric ton decrease is less than a 1% change in national emissions. This example goes to show that although methane emissions are an important agricultural issue, even a halving of methane emissions from beef cattle has a small marginal result in terms of national effects.

The above example uses only one strategy to estimate methane emissions and it is important to note that there are several equations available. The National Research Council (2016) cites the Intergovernmental Panel on Climate Change (**IPCC**) as one of the only CH_4 prediction equations tailored to predict emissions at high and low levels of dietary forage content. Under the IPCC (2006) equations, at 13 kg of intake with a gross energy concentration of 4.16 Mcal/kg, a cow on a high-forage diet would be expected to produce 264 g of CH_4 per day. On a grain-based diet consuming the same level of gross energy, emissions would be expected to be 122 g/d. Although the absolute values vary between the prediction methods, the proportional change in emissions (51% to 53%) is very similar, suggesting the net effect discussed above is supported across methane prediction approaches.

A logical next question when discussing the tradeoffs associated with the ruminant animal is more subjective—are the environmental costs worth the nutritional benefit? To address this question, it is tempting to look at the environmental impact per unit of human-edible nutrient produced; however, such an analysis fails to address the system-feasibility of meeting human requirements for particular nutrients with the food products produced in a manner divorced from the food production system. As elaborated in White and Hall (2017), comparison of the importance of different products really must be done in the context of the production system. Beef production uses byproducts from numerous other industries (dairy, corn, soy, etc.) and the question of whether the environmental costs of beef production are worth the nutritional benefit cannot be answered without more holistic, systemic assessment of these specific nutrient fluxes.

The continued search for strategies that reduce environmental impacts of beef production in an economically viable and socially acceptable manner is likely the most important way to ensure the sustainability of beef production in the United States. A number of studies have been

conducted on strategies to reduce environmental impact of beef production systems (Pelletier et al., 2010; Capper and Hayes, 2012; Stackhouse et al., 2012; Rotz et al., 2015; White et al., 2015). In general, any agricultural system with enhanced efficiency will also have improved environmental impact (Capper and Bauman, 2013). Given the structure of the beef industry in the United States, the most substantive improvements in environmental impact must be realized in the cow-calf sector (Beauchemin et al., 2010; White et al., 2014). The need for focus on the cow-calf production system presents a challenge because of the practical, economic, and social constraints placed on the cow-calf production system. More diverse than the other beef industry segments, the cow-calf sector is composed of operations varying considerably in size, profitability, and productive output. In the United States, cow-calf production is and will likely remain a forage-based enterprise. As such, improving the efficiency of cow-calf production will undoubtedly involve forage management. This will likely be challenging, however, as the cow-calf sector is generally slower at adopting new technologies and management practices. Greater extension efforts therefore should be geared toward the adoption of more efficient forage management practices. A number of reviews exist about factors influencing productivity of grazing beef cattle (Walmsley et al., 2018) and pasture and soil responses to herbivory (Drewry et al., 2008). Rather than duplicate content found in these other resources, we will focus this discussion on the implications of optimizing land use efficiency in beef production.

Figure 1 compares the ability of beef to meet the annual nutrient requirements of an average American with that of other food sources in terms of GHG emissions intensities. When evaluating nutrient output per unit of GHG input, beef does not have an advantage compared with most other animal or nonanimal products, even for those nutrients for which beef is an important source (Fig. 1). The utility of beef production is thought to be making use of land that

cannot be used for other food production endeavors. However, when the croppable land use efficiency of beef is compared to other food types (Fig. 2), more humans can be fed from other land use options. The values in Fig. 2, reflecting the number of people whose annual nutrient requirements could be met from a single acre producing each food type, are derived from White and Hall (2017) and the beef numbers may not reflect true land use efficiencies. Comparison of croppable land use efficiency is somewhat imperfect because it assumes that all croppable pasture and half of hay land is croppable and used for beef production. The estimate of 50% of hay land is based on the assumption that an average cow consumes 2% of body weight in hay for 90 d out of the year. This estimation yields an annual consumption of hay at 28.8 million tons, which is approximately 50% of the annual hay production in the U.S. in 2016 (USDA/NASS, 2018). To better understand the implications of this assumption, the analysis was duplicated assuming no hay land (15%) or all hay land (85%) should be assigned to beef cattle. What this analysis suggests is that a critical step in optimizing the role of beef production in U.S. agriculture is to find ways to eliminate competition between resources for nonanimal source food production and beef production to reinforce the role of beef cattle as a recycling engine.

GLOBAL CONSIDERATIONS FOR BEEF PRODUCTION

Beef Production Worldwide

The current largest foreign producer of beef is Brazil, which produced over 9.5 million metric tons of beef and veal in 2017. It is the world's largest exporter, with 1.86 million metric tons sold in 2017. Continued expansion of the Brazilian beef industry has been propelled by strong domestic demand and gains in important Asian markets (USDA/FAS, 2018a). China, the second-largest consumer of beef, is third in production (Zan et al., 2015). Beef demand has

increased rapidly along with the nation's economic strides. However, a depressed cattle inventory has made it impossible for the Chinese beef industry to keep pace with this demand, resulting in higher prices and an increased need to import from other countries (Li et al., 2018). India's beef and water buffalo meat (or carabeef) exports have risen substantially since the late 2000s, even surpassing Brazil in 2014. A combination of a large inventory, strong demand for cheap carabeef in poorer Asian nations, and development of the meat processing sector has fueled India's ascent to the top tier of global beef producers (Landes et al., 2016). The rebuilding of Argentine beef herds has led to gains in both the domestic market and exports. Indeed, Argentina's 2018 exports are predicted to reach a nine-year high at 350,000 tons carcass weight equivalent (USDA/FAS, 2017b). Sixth in beef production globally, Australia's severe drought conditions have resulted in considerable herd liquidation (USDA/FAS, 2018a). The duration of the current drought will most likely determine when herd rebuilding can commence. The Mexican beef industry, however, has experienced steady growth and is projected to continue this trend through its close integration with the U.S. beef industry and efforts to appeal to foreign markets (USDA/FAS, 2018c). With their financial and scientific resources, the world's top beef producers described here continue to pave the way toward more sustainable practices of beef production. These efforts are a key factor in securing the global food supply and increasing beef access for populations with serious nutrient deficiencies.

Considering the global trends in beef demand, an essential consideration for the U.S. beef industry is how to optimize its role in supplying efficiently produced beef. Beef production in the United States is, environmentally, one of the most efficient production systems in the world. Correspondingly, there is some logic to exporting U.S. beef to developing countries whose beef production systems are less environmentally efficient. However, there is concern about this

approach because it is unclear whether the export income provides sufficient societal benefit to justify the externalities associated with production. Additional work assessing this trade-off is critical to further considering the role of U.S. beef production in the growing global market.

Conclusions

Along with other top beef producers, the U.S. beef industry plays an important role in delivering essential nutrients to both domestic and foreign consumers. Beef production maintains a prominent position in the Nation's livestock industry, offering significant economic and employment opportunities. Beef cattle account for 52% of emissions from animal agriculture and 25% of all agricultural emissions in the United States (White and Hall, 2017). Enhancing resource use of beef production requires improved land management, focused on optimizing the calf weight produced per unit of land while considering conservation, biodiversity, and soil health interests. Perhaps most critically, efforts must focus on finding ways to eliminate competition between resources for nonanimal source food production and beef production to reinforce the role of beef cattle as a recycling engine. Population forecasts and environmental concerns underscore the importance of current efforts to improve productive output and sustainability of the U.S. beef industry. Indeed, the future of global food security will depend on today's decisions made regarding the direction of beef production and the food industry as a whole.

LITERATURE CITED

Ahmad, I., T. Mirza, K. Qadeer, U. Nazim, and F. H. Vaid. 2013. Vitamin B6: Deficiency diseases and methods of analysis. Pak. J. Pharm. Sci. 26:1057–1069.

Asem-Hiablie, S., T. Battagliese, K. R. Stackhouse-Lawson, and C. Alan Rotz. 2019. A life cycle assessment of the environmental impacts of a beef system in the USA. Int. J. Life Cycle Assess. 24:441–455. doi:10.1007/s11367-018-1464-6.

Bar-Shai, M., D. Gott, and S. Marmor. 2011. Acute psychotic depression as a sole manifestation of vitamin B12 deficiency. Psychosomatics. 52:384–386. doi:10.1016/j.psym.2011.01.003.

Beauchemin, K. A., H. Henry Janzen, S. M. Little, T. A. McAllister, and S. M. McGinn. 2010. Life cycle assessment of greenhouse gas emissions from beef production in western Canada: A case study. Agric. Syst. 103:371–379. doi:10.1016/j.agsy.2010.03.008.

Beckett, J. L., and J. W. Oltjen. 1993. Estimation of the water requirement for beef production in the United States. J. Anim. Sci. 71:818–826. doi:10.2527/1993.714818x.

Brundtland, G. H. 1991. Our common future. In: V. Hauff, editor. Earth and us. Oxford University Press, Oxford, United Kingdom. p. 29–31.

Capper, J. L. 2012. Is the grass always greener? Comparing the environmental impact of conventional, natural and grass-fed beef production systems. Animals. 2:127–143. doi:10.3390/ani2020127.

Capper, J. L., and D. E. Bauman. 2013. The role of productivity in improving the environmental sustainability of ruminant production systems. Annu. Rev. Anim. Biosci. 1:469–489. doi:10.1146/annurev-animal-031412-103727.

Capper, J. L., and D. J. Hayes. 2012. The environmental and economic impact of removing growth-enhancing technologies from U.S. beef production 1. J. Anim. Sci. 90:3527–3537. doi:10.2527/jas.2011-4870.

Committee on Incorporating Sustainability in the U.S. Environmental Protection Agency, Science and Technology for Sustainability Program, Policy and Global Affairs, and National Research Council. 2011. Sustainability and the U.S. EPA. National Academies Press, Washington, D.C. Available from: http://www.nap.edu/catalog/13152

Committee on Sustainability Linkages in the Federal Government, Science and Technology for Sustainability Program, Policy and Global Affairs, and National Research Council. 2013. Sustainability for the Nation: Resource connection and governance linkages. National Academies Press, Washington, D.C. Available from: http://www.nap.edu/catalog/13471

Delgado, C. L. 2003. Rising consumption of meat and milk in developing countries has created a new food revolution. J. Nutr. 133:3907S-3910S. doi:10.1093/jn/133.11.3907S.

DiNicolantonio, J. J., M. F. McCarty, and J. H. OKeefe. 2018. Role of dietary histidine in the prevention of obesity and metabolic syndrome. Open Heart. 5:e000676. doi:10.1136/openhrt-2017-000676.

Drewry, J. J., K. C. Cameron, and G. D. Buchan. 2008. Pasture yield and soil physical property responses to soil compaction from treading and grazing—a review. Soil Res. 46:237–256. doi:10.1071/SR07125.

Ellis, J. L., A. Bannink, J. France, E. Kebreab, and J. Dijkstra. 2010. Evaluation of enteric methane prediction equations for dairy cows used in whole farm models. Glob. Change Biol. 16:3246–3256. doi:10.1111/j.1365-2486.2010.02188.x.

Gille, D., and A. Schmid. 2015. Vitamin B12 in meat and dairy products. Nutr. Rev. 73:106–115. doi:10.1093/nutrit/nuu011.

Gomiero, T., D. Pimentel, and M. G. Paoletti. 2011. Is there a need for a more sustainable agriculture? Crit. Rev. Plant Sci. 30:6–23. doi:10.1080/07352689.2011.553515.

Green, R., L. H. Allen, A.-L. Bjørke-Monsen, A. Brito, J.-L. Guéant, J. W. Miller, A. M. Molloy, E. Nexo, S. Stabler, B.-H. Toh, P. M. Ueland, and C. Yajnik. 2017. Vitamin B12 deficiency. Nat. Rev. Dis. Primer. 3:1–20. doi:10.1038/nrdp.2017.40.

Hertel, T. W. 2011. The global supply and demand for agricultural land in 2050: A perfect storm in the making? Am. J. Agric. Econ. 93:259–275. doi:10.1093/ajae/aaq189.

Holden, J. M., S. Gebhard, C. S. Davis, and D. G. Lurie. 1991. A nationwide study of the selenium contents and variability in white bread. J. Food Compos. Anal. 4:183–195. doi:10.1016/0889-1575(91)90030-A.

Intergovernmental Panel on Climate Change. 2006. 2006 IPCC guidelines for national greenhouse gas inventories. Institute for Global Environmental Strategies, Hayama, Kanagawa, Japan. Available from: http://www.ipcc-nggip.iges.or.jp/public/2006gl/index.htm

Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483–2492. doi:10.2527/1995.7382483x.

Landes, M., A. Melton, and S. Edwards. 2016. India emerges as a major beef exporter. USDA Econ. Res. Serv. Available from: https://www.ers.usda.gov/amber-waves/2016/july/ india-emerges-as-a-major-beef-exporter/

Lawrence, G. D. 2013. Dietary fats and health: Dietary recommendations in the context of scientific evidence. Adv. Nutr. 4:294–302. doi:10.3945/an.113.003657.

Li, X. Z., C. G. Yan, and L. S. Zan. 2018. Current situation and future prospects for beef production in China — A review. Asian-Australas. J. Anim. Sci. 31:984–991. doi:10.5713/ajas.18.0212.

Mader, T. L., and M. S. Davis. 2004. Effect of management strategies on reducing heat stress of feedlot cattle: Feed and water intake. J. Anim. Sci. 82:3077–3087. doi:10.2527/2004.82103077x.

Martens, J. H., H. Barg, M. J. Warren, and D. Jahn. 2002. Microbial production of vitamin B 12. Appl. Microbiol. Biotechnol. 58:275–285. doi:10.1007/s00253-001-0902-7.

Martínez, Y., X. Li, G. Liu, P. Bin, W. Yan, D. Más, M. Valdivié, C.-A. A. Hu, W. Ren, and Y. Yin. 2017. The role of methionine on metabolism, oxidative stress, and diseases. Amino Acids. 49:2091–2098. doi:10.1007/s00726-017-2494-2.

Mekonnen, M. M., and A. Y. Hoekstra. 2012. A global assessment of the water footprint of farm animal products. Ecosystems. 15:401–415. doi:10.1007/s10021-011-9517-8.

Mills, J. A. N., E. Kebreab, C. M. Yates, L. A. Crompton, S. B. Cammell, M. S. Dhanoa, R. E. Agnew, and J. France. 2003. Alternative approaches to predicting methane emissions from dairy cows. J. Anim. Sci. 81:3141–3150. doi:10.2527/2003.81123141x.

Mitloehner, F. 2018. Livestock and climate change: Facts and fiction. In: T. Engle, D. J. Klingborg, and B. E. Rollin, editors. The welfare of cattle. CRC Press, Boca Raton, FL. p. 27–30.

Moe, P. W., and H. F. Tyrrell. 1979. Methane production in dairy cows. J. Dairy Sci. 62:1583–1586.

Nardone, A., B. Ronchi, N. Lacetera, M. S. Ranieri, and U. Bernabucci. 2010. Effects of climate changes on animal production and sustainability of livestock systems. Livest. Sci. 130:57–69. doi:10.1016/j.livsci.2010.02.011.

Nguyen, T. L. T., J. E. Hermansen, and L. Mogensen. 2010. Environmental consequences of different beef production systems in the EU. J. Clean. Prod. 18:756–766. doi:10.1016/j.jclepro.2009.12.023.

NRC. 2016. Nutrient requirements of beef cattle. 8th ed. National Academies Press, Washington, DC.

OECD and FAO. 2014. Beef and veal projections: Production and trade. In: Agricultural outlook. 20th ed. OECD, Paris, France. Available from: https://www.oecd-ilibrary.org/agriculture-and-food/oecd-fao-agricultural-outlook-2014_agr_outlook-2014-en

Oltjen, J. W., and J. L. Beckett. 1996. Role of ruminant livestock in sustainable agricultural systems. J. Anim. Sci. 74:1406–1409. doi:10.2527/1996.7461406x.

Pawlak, R., S. J. Parrott, S. Raj, D. Cullum-Dugan, and D. Lucus. 2013. How prevalent is vitamin B12 deficiency among vegetarians? Nutr. Rev. 71:110–117. doi:10.1111/nure.12001.

Pelletier, N., R. Pirog, and R. Rasmussen. 2010. Comparative life cycle environmental impacts of three beef production strategies in the Upper Midwestern United States. Agric. Syst. 103:380–389. doi:10.1016/j.agsy.2010.03.009.

Rotz, C. A., S. Asem-Hiablie, J. Dillon, and H. Bonifacio. 2015. Cradle-to-farm gate environmental footprints of beef cattle production in Kansas, Oklahoma, and Texas. J. Anim. Sci. 93:2509–2519. doi:10.2527/jas.2014-8809.

Seale, J. L. 1998. Changes in the structure of global food demand: Discussion. Am. J. Agric. Econ. 80:1062–1063. doi:10.2307/1244204.

Solomons, N. W. 2001. Dietary sources of zinc and factors affecting its bioavailability. Food Nutr. Bull. 22:138–154. doi:10.1177/156482650102200204.

Speer, N. 2018. Beef exports versus imports; the numbers tell a positive tale. Beef Mag. Available from: https://www.beefmagazine.com/exports/beef-exports-versus-imports-numbers-tell-positive-tale

Stackhouse, K. R., C. A. Rotz, J. W. Oltjen, and F. M. Mitloehner. 2012. Growth-promoting technologies decrease the carbon footprint, ammonia emissions, and costs of California beef production systems. J. Anim. Sci. 90:4656–4665. doi:10.2527/jas.2011-4654.

Stewart, R. L., R. E. Silcox, C. Lacy, D. W. Hancock, G. H. Harris, and R. W. Ellis. 2012. Improve grazing management, increase forage use efficiency. Progress. Forage. 31. Available from: https://www.progressiveforage.com/forage-types/grassesand- grazing/improve-grazing-management-increaseforage- use-efficiency

Surjana, D., G. M. Halliday, and D. L. Damian. 2010. Role of nicotinamide in DNA damage, mutagenesis, and DNA repair. J. Nucleic Acids. 2010:1–13. doi:10.4061/2010/157591.

Tanphaichitr, V., and H. P. Broquist. 1973. Role of lysine and -N-trimethyllysine in carnitine biosynthesis. II. Studies in the rat. J. Biol. Chem. 248:2176–2181.

Thiry, C., A. Ruttens, L. De Temmerman, Y.-J. Schneider, and L. Pussemier. 2012. Current knowledge in species-related bioavailability of selenium in food. Food Chem. 130:767–784. doi:10.1016/j.foodchem.2011.07.102.

United Nations, Department of Economic and Social Affairs. 2017. World population prospects: The 2017 revision.

United States Environmental Protection Agency (EPA). 2018. Inventory of U.S. greenhouse gas emissions and sinks. Createspace Independent Publishing Platform, Scotts Valley, CA.

US Meat Export Federation. 2017. Total U.S. beef exports 2008 to 2017 (including variety meat). Available from: http://www.usmef.org/ news-statistics/statistics/

US Meat Export Federation. 2018. Tremendous first half for U.S. beef exports; pork exports still ahead of 2017 pace. Available from: http://www.usmef.org/news-statistics/press-releases/ tremendous-first-half-for-u-s-beef-exports-pork-exportsstill- ahead-of-2017-pace/

USDA Economic Research Service. 2018a. Statistics and information. Available from: https://www.ers.usda.gov/topics/animal-products/cattlebeef/ statistics-information/

USDA Economic Research Service. 2018b. Red meat and poultry production. Available from: https://www.ers.usda.gov/dataproducts/ livestock-meat-domestic-data/livestock-meatdomestic-data/#Red%20meat%20and%20poultry%20 production

USDA Economic Research Service. 2018c. Annual cash receipts by commodity. Available from: https://data.ers.usda.gov/reports. aspx?ID=17832

USDA Foreign Agricultural Service. 2017a. In China, Perdue welcomes U.S. beef back to market. Available from: https://www.fas.usda. gov/newsroom/china-perdue-welcomes-us-beef-backmarket

USDA Foreign Agricultural Service. 2017b. Argentina: Livestock and products annual. Available from: https://www.fas.usda.gov/data/ argentina-livestock-and-products-annual-2

USDA Foreign Agricultural Service. 2018a. Livestock and poultry: World markets and trade. Available from: https://apps.fas.usda. gov/psdonline/circulars/livestock_poultry.pdf

USDA Foreign Agricultural Service. 2018b. Partnering to promote U.S. beef to growing Indonesian market. Available from: https://www. fas.usda.gov/newsroom/partnering-promote-us-beef-growingindonesian- market

USDA Foreign Agricultural Service. 2018c. Mexico: Livestock and products annual. Available from: https://www.fas.usda.gov/data/mexicolivestock- and-products-annual-4

USDA Forest Service. 2018. Rangeland management. Available from: https://www.fs.fed.us/rangeland-management/aboutus/index.shtml

USDA National Agricultural Statistics Service. 2012. Census of agriculture. Available from: https://www.nass.usda.gov/Publications/ AgCensus/2012/Full_Report/Volume_1,_Chapter_1_US/ st99_1_001_001.pdf

USDA National Agricultural Statistics Service. 2013. Census of agriculture: 2013 farm and ranch irrigation survey. Available from: https://www.nass.usda.gov/Publications/AgCensus/2012/ Online_Resources/Farm_and_Ranch_Irrigation_Survey/

USDA National Agricultural Statistics Service. 2018. QuickStats. Available from: https://quickstats.nass.usda.gov/

USDL Bureau of Labor Statistics. 2014. Employment by detailed occupation. Available from: https://www.bls.gov/emp/ep_table_102. htm

Uzel, C., and M. E. Conrad. 1998. Absorption of heme iron. Semin. Hematol. 35:27–34.

Vahmani, P., C. Mapiye, N. Prieto, D. C. Rolland, T. A. McAllister, J. L. Aalhus, and M. E. R. Dugan. 2015. The scope for manipulating the polyunsaturated fatty acid content of beef: A review. J. Anim. Sci. Biotechnol. 6:29. doi:10.1186/s40104-015-0026-z.

Van Der Torre, H. W., W. V. Dokkum, G. Schaafsma, M. Wedel, and T. Ockhuizen. 1991. Effect of various levels of selenium in wheat and meat on blood Se status indices and on Se balance in Dutch men. Br. J. Nutr. 65:69–80. doi:10.1079/BJN19910067.

Wallace, J. S. 2000. Increasing agricultural water use efficiency to meet future food production. Agric. Ecosyst. Environ. 82:105–119. doi:10.1016/S0167-8809(00)00220-6.

Walmsley, B. J., S. J. Lee, P. F. Parnell, and W. S. Pitchford. 2018. A review of factors influencing key biological components of maternal productivity in temperate beef cattle. Anim. Prod. Sci. 58:1. doi:10.1071/AN12428.

White, R. R., M. Brady, J. L. Capper, and K. A. Johnson. 2014. Optimizing diet and pasture management to improve sustainability of U.S. beef production. Agric. Syst. 130:1–12. doi:10.1016/j.agsy.2014.06.004.

White, R. R., M. Brady, J. L. Capper, J. P. McNamara, and K. A. Johnson. 2015. Cow–calf reproductive, genetic, and nutritional management to improve the sustainability of whole beef production systems. J. Anim. Sci. 93:3197–3211. doi:10.2527/jas.2014-8800.

White, R. R., and J. L. Capper. 2013. An environmental, economic, and social assessment of improving cattle finishing weight or average daily gain within U.S. beef production. J. Anim. Sci. 91:5801–5812. doi:10.2527/jas.2013-6632.

White, R. R., and M. B. Hall. 2017. Nutritional and greenhouse gas impacts of removing animals from US agriculture. Proc. Natl. Acad. Sci. 114:E10301–E10308. doi:10.1073/pnas.1707322114.

Winchester, C. F., and M. J. Morris. 1956. Water intake rates of cattle. J. Anim. Sci. 15:722–740. doi:10.2527/jas1956.153722x.

Zan, L. S., C. G. Mei, and H. C. Wang. 2015. Current situation, existing problems and countermeasures on development of beef cattle industry in China. J Northwest AF Univ. 6:48–52.

TABLES

Table 1. Contributions of beef cattle to U.S. nutrient supplies

Nutrient	Human Nutrient Requirement Years Supplied, millions[1]	Percent of Total U.S. Supply[2]	Percent of Total U.S. Requirement[3]
Energy	14.9	2.30	4.70
Protein	43.1	4.98	13.62
Linoleic acid	5.0	0.75	1.57
alpha-Linolenic acid	20.4	3.31	6.45
Calcium	0.8	0.24	0.26
Iron	19.5	2.66	6.17
Magnesium	5.8	0.75	1.84
Phosphorus	23.3	1.97	7.38
Potassium	6.7	1.98	2.12
Zinc	45.8	6.58	14.50
Copper	9.4	0.87	2.96
Selenium	35.3	2.49	11.15
Thiamin	8.5	0.89	2.68
Riboflavin	17.4	2.19	5.50
Niacin	28.7	4.05	9.07
Folate	2.1	0.46	0.68
Vitamin B6	29.0	3.32	9.17
Vitamin B12	137.0	15.53	43.33
Cysteine	55.6	3.67	17.58
Histidine	69.9	5.79	22.11
Isoleucine	50.9	5.15	16.09
Leucine	45.3	4.70	14.33
Lysine	58.9	7.04	18.64
Methionine	50.8	5.74	16.06
Phenylalanine or Tyrosine	32.2	4.00	10.19
Threonine	60.3	5.62	19.08
Tryptophan	61.3	4.91	19.38
Valine	43.1	4.63	13.65
EPA+DHA	47.0	10.45	14.85
AA	486.6	7.52	153.93

[1]Value indicates the number of people (millions) whose annual requirement of each nutrient would be supplied by beef.

[2]Value indicates the percent of the total amount of each nutrient supplied by the U.S. agricultural system coming from beef.

[3]Value indicates the percent of the total U.S. population's requirement for each nutrient that would be satisfied from beef.

Table 2. Major importers of U.S. beef and beef variety meats in 2017[1]

Market	Import volume, metric tons	Dollar value, millions
Japan	307,559	1,890
Mexico	237,972	980
South Korea	184,152	1,220
Hong Kong	130,726	884
Canada	116,561	796
Taiwan	44,800	410

[1]Adapted from USMEF (2017).

FIGURES

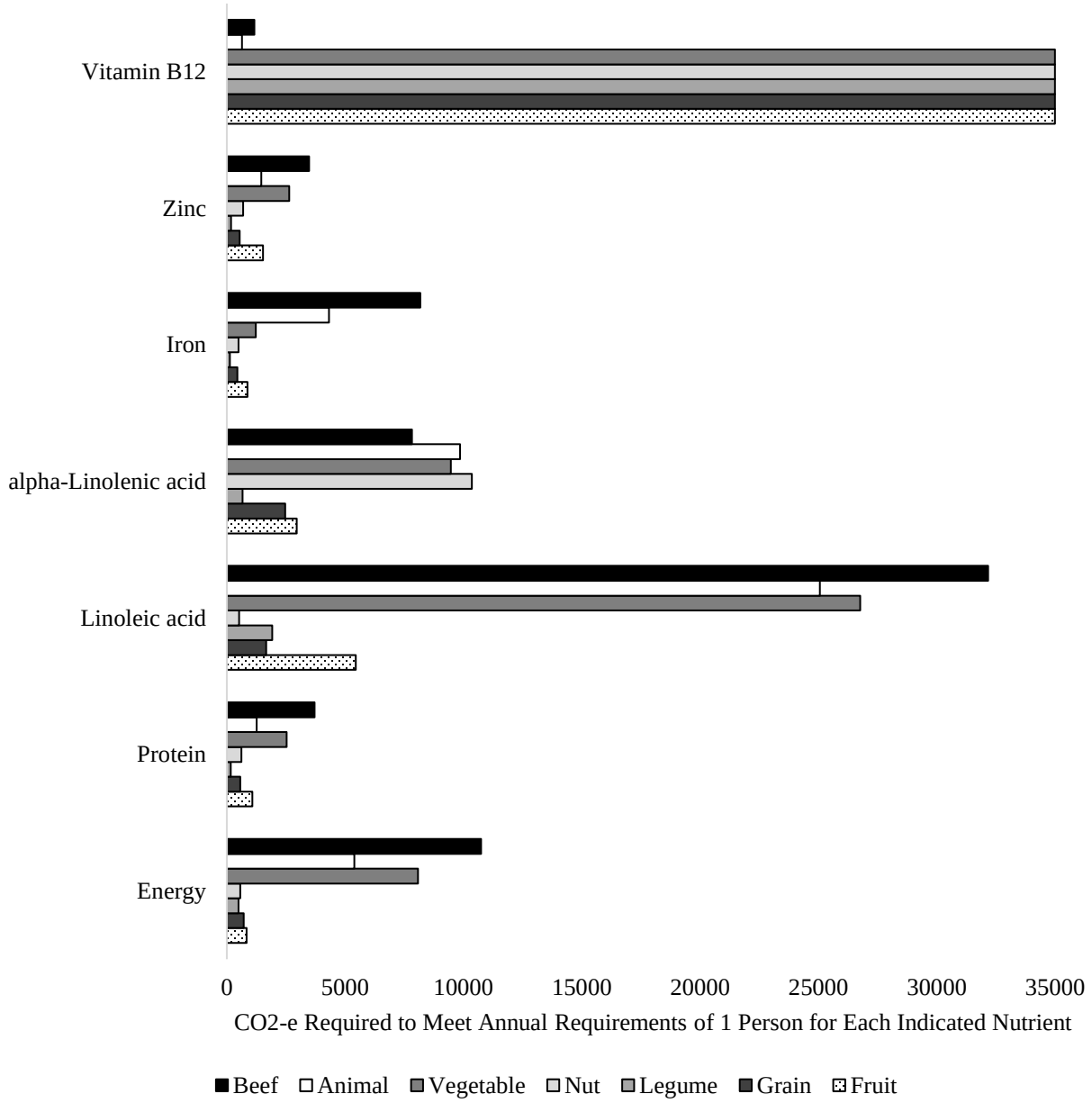

Figure 1. Greenhouse gas emissions intensities of producing sufficient product (beef, animal products, vegetables, nuts, legumes, grains, or fruits) to meet the nutrient (vitamin B12, zinc, iron, alpha-linolenic acid, linoleic acid, protein, and energy) requirements of an average American for 1 yr. Animal products include eggs/poultry, fish/mollusks, lamb/mutton/goat, pork, and milk. Arachadonic acid and EPD+DHA results are not shown due to lack of concentrations in nonanimal source products.

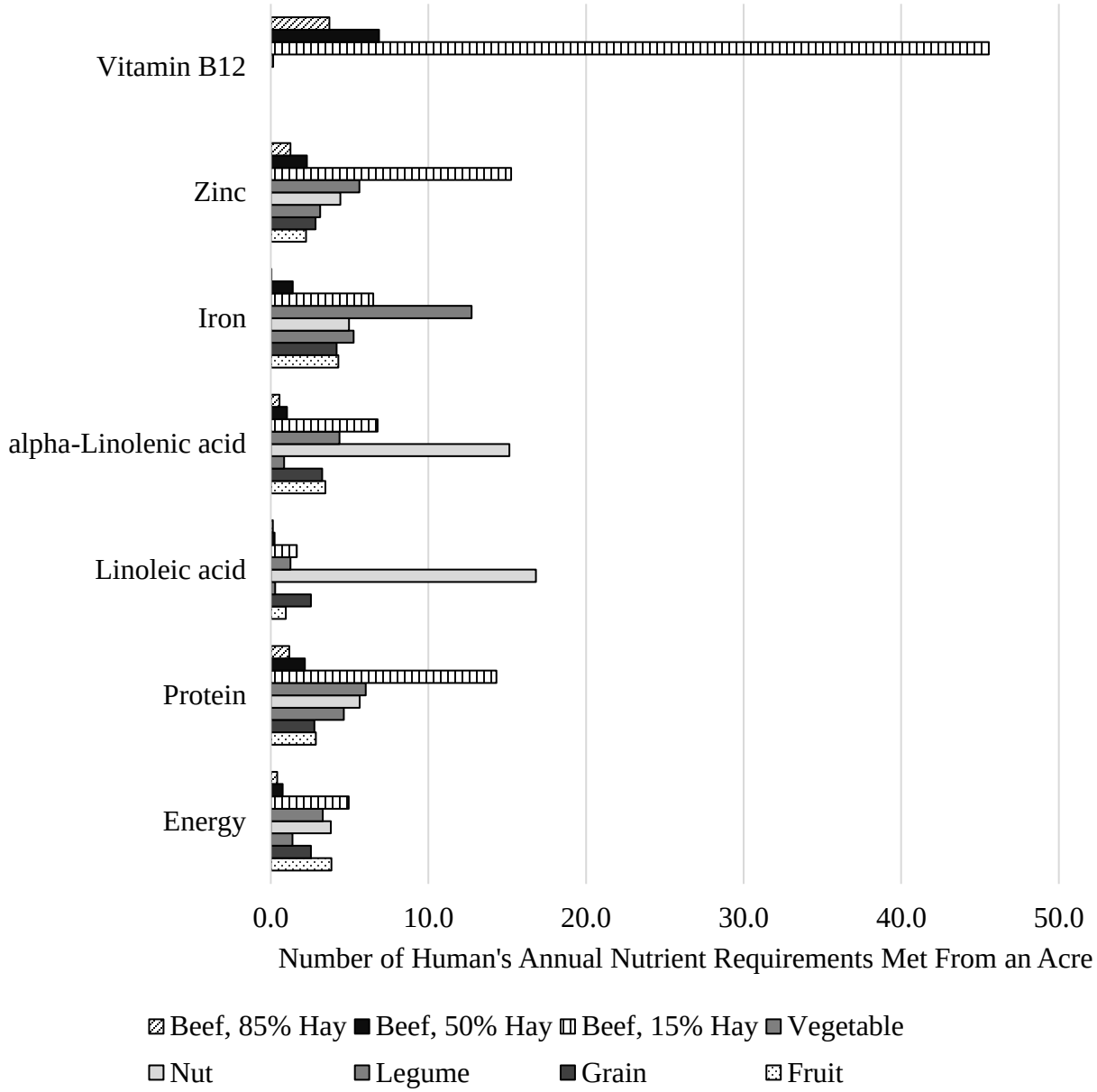

Figure 2. The number of average Americans whose yearly nutrient requirements could be met from an acre of land producing each food type. Opportunities to feed people through beef production were calculated assuming 15% of hay land, 50% or hay land, or 85% of hay land were attributable to beef production. Arachadonic acid and EPD+DHA results are not shown due to lack of concentrations in nonanimal source products.

CHAPTER 2: Literature Review

FEED ANALYSIS

Determination of Nutrient Content

Nutrient content determination of feeds is an essential foundation of animal nutrition. Advances in nutrition are largely reliant on analytical methods that can accurately assess a feed's chemical composition (Ensminger et al., 1990). Additionally, information on the levels of nutrients a feed contains is needed for formulation of balanced diets intended to meet the nutrient requirements of livestock. Close adherence to accepted analytical methods is essential to allow for comparison of results from different nutrition research studies. Major laboratory analyses of a feed generally include assessing the percentage of dry matter, ash, neutral detergent fiber, acid detergent fiber, starch, and crude protein it contains, a process referred to as proximate analysis (Pond et al., 2005).

Dry Matter

Estimation of dry matter (**DM**) content of feeds is the most basic and probably the most common analysis performed in nutrition laboratories. The amount of moisture present in feedstuffs can vary greatly due to feed type, environmental conditions, and storage method (Pond et al., 2005). Accounting for this moisture is therefore essential before comparisons between different feeds can be made (Pond et al., 2005). As a standard practice in ruminant nutrition, the nutrient content of feeds is always expressed on a DM basis. The simplest method to determine DM content involves weighing the ground feed sample, placing it in a heat-resistant container, and drying it in a forced-air oven to evaporate all moisture (Pond et al., 2005). The percent DM content is then found by dividing the weight of the dried residue by the weight of the original sample and multiplying by 100.

For samples that are less than approximately 85% DM in their as-fed (**AF**) state, partial drying to 3-15% moisture is necessary before grinding in a laboratory mill (Undersander et al., 1993). According to Goering and Van Soest (1970), feeds such as silage, haylage, and high-moisture corn grain should be partially dried in a forced-air oven at 55°C for 16-24 hours (Goering and Van Soest, 1970). They state that temperature should not exceed 60°C as high temperatures can elicit chemical changes that may impact the results of neutral detergent fiber, acid detergent fiber, and lignin analyses. The sample may then be ground and subjected to nutrient analyses.

Guidelines set by the Association of Official Analytical Chemists (**AOAC**) state that total DM of ground samples should be obtained by drying in a forced-air oven at 135°C for 2 hours, which removes virtually all moisture (AOAC, 2000). Total DM may also be assessed by oven drying the ground sample at 100°C for 24 hours or 105°C for 16 hours, as stated by Undersander et al. (1993). After drying, DM content is calculated as:

$$\%DM = ((Wt3 - Wt1) / (Wt2 - Wt1)) \times 100$$

where

Wt1 = tare weight of container in grams

Wt2 = initial weight of sample and container in grams

Wt3 = dry weight of sample and container in grams

The %DM may then be used to correct other nutrient analysis values for moisture by dividing the nutrient value by the %DM. The nutrient value is now said to be expressed on a DM basis and more direct comparisons with other feeds can be made.

Ash

When all combustible material in a feed sample has been completely burned off, or oxidized, the remaining inorganic residue is termed *ash*. Exceptionally high values of ash may indicate soil contamination of the feed (Pond et al., 2005). A straightforward process, ashing a ground feed sample involves placing the sample in a porcelain crucible after obtaining its weight, then igniting it in a muffle furnace (Pond et al., 2005). After removal from the furnace and cooling to room temperature, the weight of the remaining residue is divided by that of the original sample to obtain the ash content using the formula

%ash = ashed sample wt (g)/unashed sample wt (g) x 100

According to AOAC guidelines, the sample should be maintained at a temperature of 600°C for 2 hours minimum inside the furnace (AOAC, 2000). Ensminger et al. (1990) and Pond et al. (2005) both stated that a temperature range of 500 to 600°C was appropriate for ashing but failed to specify a length of time (Ensminger et al., 1990; Pond et al., 2005). Peacock (1992) stated a minimum temperature of 500°C and explained that a typical muffle furnace takes approximately 5 hours to reach this temperature (Peacock, 1992). Complete ashing of the sample is then achieved after 13 hours of exposure to 500°C (Peacock, 1992).

In order to assess the amounts of individual minerals in a sample after ashing, a variety of analytical techniques have been employed, including atomic absorption (David, 1959; Cresser and Parsons, 1979), flame emission spectroscopy (Cresser and Parsons, 1979), inductively coupled plasma spectrometry (Munter et al., 1979; Zarcinas et al., 1987), and x-ray fluorescence (Norrish and Hutton, 1977). Prior to analysis, the ashed sample must be digested in strong acid to dissolute the mineral components and deliver them in a clear aqueous form. According to

Gorsuch (1959), mixtures of nitric and perchloric acids or nitric, perchloric, and sulfuric acids result in satisfactory digestion of the mineral elements (Gorsuch, 1959). Munter (1979) and Zarcinas et al. (1987) also utilized nitric-perchloric digestion with success (Munter et al., 1979; Zarcinas et al., 1987).

Fiber

The cell walls of plants are composed of various structural polysaccharides crosslinked with phenolic compounds and proteins (Hindrichsen et al., 2006). The 2 main structural polysaccharides include cellulose and hemicellulose, and lignin is the major phenolic compound involved in crosslinking with cellulose and hemicellulose (Bidlack, 1992; NRC, 2016). Cellulose is composed of linear chains of D-glucose molecules connected by β-(1,4) linkages. Parallel chains are linked with hydrogen bonds and van der Waals forces, which form tubular structures termed microfibrils (Bidlack, 1992). These microfibrils are linked to hemicellulose with hydrogen bonds. Hemicellulose represents a group of polysaccharides defined as having backbones of glucose, mannose, and xylose joined with β-(1,4) linkages (NRC, 2016). Hemicellulose is connected to lignin via ester and ether bonding. Lignin is a polymer composed of guaiacylpropane, syringylpropane, and phydroxyphenylpropane subunits (Chen, 2014). It serves to anchor the microfibrils and other matrix polysaccharides in a stiff complex, thereby lending strength to the cell wall and protection against degradation (Chen, 2014). To the ruminant animal, cellulose is highly digestible and hemicellulose is partially digestible while lignin is indigestible and hampers the digestibility of other fractions (Ensminger et al., 1990). Collectively, the 3 components represent *fiber*, a major portion of the ruminant diet and the primary source of digestible energy for virtually all ruminants (Ensminger et al., 1990).

Crude fiber (**CF**), or Weende cellulose, is composed of cellulose and lignin and is intended to indicate the relative indigestibility of a feed sample (Ensminger et al., 1990; Jung, 1997). Developed by Henneberg and Stohmann in 1859, the Weende method of fiber extraction involves digesting the feed sample in dilute acid followed by digestion in dilute base (Henneberg and Stohmann, 1859). The insoluble residue left over is then filtered, dried, and ashed in a furnace and the CF content is represented by the initial residue weight minus the weight of the ash (Ensminger et al., 1990; Pond et al., 2005) Although in use for many decades, the popularity of the Weende method has declined substantially because many researchers have attested to its limitations (Norman, 1935; Williams and Olmsted, 1935; Bredon and Juko, 1961; Van Soest and McQueen, 1973; Van Soest, 1994). The major limitations of CF analysis are (1) compositional variability of the product and (2) poor assessment of overall feed digestibility due to differing digestibilities between fiber types. Bredon and Juko (1961) explained that, although the CF fraction obtained is predominantly composed of cellulose and lignin, the amounts of these components are not necessarily reflective of those present in the original sample (Bredon and Juko, 1961). Norman (1935) for instance described dramatic variation in component percentages recovered, with 40 to 88% given for cellulose and 4 to 67% for lignin (Norman, 1935). Van Soest and McQueen (1973) cited a similar range for lignin recovery at 10 to 50% (Van Soest and McQueen, 1973). The often severe underestimation of lignin by the Weende method is due to the fact that lignin may end up in other chemical fractions (such as ash) during analysis (Ensminger et al., 1990; Van Soest, 1994). Crude fiber with lower lignin content therefore may not necessarily be from the less lignified source. Crude fiber is also considered a poor estimator of digestibility because the fiber in young plants is mostly cellulose while the fiber in mature plants is represented by increased lignin (Ensminger et al., 1990). As stated earlier, lignin is indigestible

to ruminants while cellulose is highly digestible. The Weende method is not capable of distinguishing between these 2 components and therefore cannot provide a reliable assessment of digestibility (Lloyd et al., 1978; Ensminger et al., 1990).

In light of the Weende method's inadequacies, Van Soest and colleagues developed detergent-based procedures to estimate not only the total cell wall content but also the individual fractions of cellulose, hemicellulose, and lignin present in feedstuffs (Van Soest, 1963; Van Soest and Wine, 1967; Van Soest et al., 1991). The fiber residues yielded by these methods are termed neutral detergent fiber (**NDF**), acid detergent fiber (**ADF**), and acid detergent lignin (**ADL**). Composed of cellulose, hemicellulose, and lignin, NDF is a representation of the total cell wall content. It is considered an estimator of feed intake because it contains the slowly digested fibrous fractions that take up space in the rumen (Ensminger et al., 1990). High NDF content therefore leads to decreased voluntary intake and vice versa (Ensminger et al., 1990) Acid detergent fiber contains cellulose and lignin, but no hemicellulose. As such, ADF is most comparable to CF and is considered an improved indicator of forage digestibility (Van Soest, 1963; Ensminger et al., 1990; Pond et al., 2005)Subjecting ADF residue to sulfuric acid yields ADL, which represents the (indigestible) lignin content. The improved accuracy, reliability, and informativeness of Van Soest fiber analysis over Weende fiber analysis has resulted in a considerable shift in preference toward the former among the animal and food science communities.

The philosophy of the Van Soest NDF analysis procedure is to utilize a detergent solution of neutral pH to isolate the plant cell wall contents by extracting non-structural carbohydrates, lipids, protein, non-protein nitrogen, organic acids, pectin, tannin, and silica (Van Soest and Wine, 1967; Ensminger et al., 1990; Pond et al., 2005). The original NDF procedure involved

boiling the feed sample with neutral detergent solution (containing sodium lauryl sulfate), decahydronaphthalene, and sodium sulfite in a refluxing apparatus for 1 hour (Van Soest and Wine, 1967). After rinsing, soaking in acetone, and drying, the remaining residue was said to represent the cell wall constituents. In a later publication, Van Soest and colleagues (1991) declared the original procedure obsolete and put forth a number of updates intended to improve and standardize the procedure (Van Soest et al., 1991). Their first recommendation was to utilize heat stable α-amylase, an enzyme responsible for starch degradation, explaining that the original procedure was initially applied to forages and its subsequent use on samples with elevated starch content demonstrated interference by starch. The attempts of other researchers to combat this issue had led to the use of various amylases which resulted in inconsistent results (Van Soest et al., 1991). The use of sodium sulfite was also encouraged as a technique to reduce protein residue in NDF since sodium sulfite is capable of dissolving crosslinked proteins (Van Soest et al., 1991). However, it was recommended that sodium sulfite be omitted in cases where ADL would ultimately be determined as the sodium sulfite may also degrade lignin. The third major update was replacement of ethylene glycol with triethylene glycol in the NDF solution due to health issues associated with the former. A criticism of the NDF method is that, while NDF is said to represent the fiber fraction of feed, pectinic substances of the cell wall become solubilized during reflux and therefore lead to an underestimation of cell wall content (Van Soest, 1994; Jung, 1997). This is certainly an issue if the goal of analysis is to study the structural biology of plant cells but should not be of concern in cases where fiber is defined as the incompletely digestible fraction of a feed (Van Soest, 1994). As explained by Van Soest, pectin is considered part of the cell wall matrix but differs from the other fibrous fractions in that it is completely fermented in the rumen (Van Soest, 1994).

The procedure to determine ADF content is similar in many ways to NDF analysis, where a ground feed sample is boiled with detergent for 1 hour in a reflux apparatus to remove the nonstructural components of the plant cell. The remaining residue is rinsed, soaked in acetone, and dried, and residue weight is then used to calculate %ADF of the original sample. Prepared by mixing cetyl trimethylammonium bromide and 0.5M sulfuric acid, the ADF solution extracts components similarly to the NDF solution but also solubilizes hemicellulose, which is the distinguishing factor between NDF and ADF (Van Soest, 1963; AOAC, 2000). According to Van Soest and Robertson (1980), one may perform ADF analysis on a feed sample that has already been put through the NDF procedure, providing an opportunity to increase analytical efficiency (Van Soest and Robertson, 1980). In addition to being its own fiber analysis procedure, determination of ADF is a necessary step in the procedure for ADL determination (Van Soest, 1963). Residue left from ADF analysis is soaked in 72% sulfuric acid, which dissolves the cellulose and leaves lignin content intact. After 3 hours, the remaining residue is rinsed with hot water until acid-free, then dried and ashed in a muffle furnace. Acid detergent lignin content is calculated as

$$(L \times 100)/S$$

where L = loss upon ashing after 72% sulfuric acid treatment and S = weight of oven dried sample.

Starch

Starch serves as the predominant form of carbohydrate storage in plants (NRC, 2016). It is composed of repeating units of glucose molecules linked by glycosidic bonds that occur as

linear structures, termed amylose, or branched structures, termed amylopectin (Gallant et al., 1992). Starch is most abundant in cereal grains, where it resides in granules inside a nutritive tissue referred to as the endosperm (Baroux et al., 2002; NRC, 2016). Inside each granule, amylopectin is present as a helical crystalline system with amylose dispersed throughout (Gallant et al., 1992; Knudsen, 1997). Starch is an important component of livestock feeds and is frequently incorporated in ration formulation (Hall, 2009). Starch concentrations in feeds are generally determined using enzymatic methods that are able to differentiate the glucose in starch from glucose in other carbohydrate fractions (Hall et al., 2001). Total hydrolysis of the starch granule components (amylose and amylopectin) as well as an accurate assessment of the amount of glucose released are critical to the success of the starch analysis method (Karkalas, 1985; Hall et al., 2001). The most current and broadly utilized assay following AOAC guidelines involves first incubating dried and ground feed samples with alpha-amylase enzyme in sodium acetate buffer solution (pH 5.0) at 100°C for 1 h (Hall, 2015). This step accomplishes gelatinization and partial hydrolysis of the starch content. The sample is then incubated with amyloglucosidase enzyme at 50°C for 2 h (Hall, 2015). Amyloglucosidase serves to hydrolyze short-chain dextrins to free glucose molecules (Thivend et al., 1972). After the addition of water, mixing, and clarification, the quantity of free glucose is then determined by way of a colorimetric glucose oxidase-peroxidase assay (Karkalas, 1985; Hall, 2015)d by glucose oxidase enzyme into gluconic acid and hydrogen peroxide (Karkalas, 1985). Peroxidase enzyme then degrades the hydrogen peroxide in the presence of a chromogen, typically 4-aminoantipyrine, to yield a light-absorbing complex to which colorimetric analysis may then be applied (Porstmann et al., 1981; Karkalas, 1985). A pure glucose sample is also run with the feed samples for the entirety of the assay and is used to create a standard curve (Hall, 2003). The sample starch percentage is

calculated from the absorbances using the linear and quadratic coefficients of the standard curve as follows:

Starch, % = abs × linear coefficient + abs^2 × quadratic coefficient

where *abs* equals the blank corrected absorbance of the sample. The starch percentage is finally corrected for the volume of fluid used in the analysis and sample absolute dry matter content (Hall, 2015).

Crude Protein

High protein content increases the nutritional and economical values of a feed product (Brady and Clauser, 1991), making it an important nutrient to analyze. Crude protein (**CP**) represents the approximate amount of protein in feed that is determined by multiplying the feed's nitrogen content by a conversion factor of 6.25, which assumes plant proteins are 16% nitrogen by weight, on average (Khanizadeh et al., 1995; Pond et al., 2005). The longstanding method of CP analysis, the Kjeldahl method, was developed by chemist Johan Kjeldahl in 1883 (Kjeldahl, 1883; Ensminger et al., 1990). Samples are first digested in concentrated sulfuric acid in the presence of a metal catalyst to release the nitrogen as ammonium (Kjeldahl, 1883; Bradstreet, 1954; Sáez-Plaza et al., 2013). Catalysts most commonly used have included mercury, copper, potassium, and selenium (Bradstreet, 1954; Hansen, 1989; Marcó et al., 2002). The digested solution is then alkalinized, distilled, and titrated to measure ammonia content (Bradstreet, 1954; Sáez-Plaza et al., 2013). An alternative to the Kjeldahl method, the Dumas (or combustion) method, analyzes feed nitrogen content through quantification by thermal conductivity (Sweeney and Rexroad, 1987; Hansen, 1989). In the Dumas method, the sample is completely burned in an induction furnace to release nitrogen gases (Sweeney and Rexroad, 1987; Miller et al., 2007;

Sáez-Plaza et al., 2013). Any nitrogen oxides are then reduced to nitrogen and the nitrogen content is quantified using thermal detection (Sweeney and Rexroad, 1987; Miller et al., 2007). Advantages of the Dumas method over the Kjeldahl method are that it is less time- and labor-intensive and does not require hazardous chemicals (Rhee, 2001; Sáez-Plaza et al., 2013). Studies comparing the Kjeldahl and Dumas methods have reported comparable results between the two (Etheridge et al., 1998; Marcó et al., 2002) or slight superiority of the Dumas method in terms of accuracy and precision (Schmitter and Rihs, 1989; Miller et al., 2007). Miller and colleagues (2007) suggested that the difference may be due to incomplete digestion of certain nitrogenous components, specifically tryptophan and nicotinic acid, and variability between different catalysts used in the Kjeldahl method. The advantages of the Dumas method, coupled with a growing demand for feed CP analysis, have resulted in gradual replacement of the Kjeldahl method in many laboratories (Simonne et al., 1997; Etheridge et al., 1998).

GENERAL RUMINANT NUTRITION

The Polygastric System

The major digestive organ of ruminant animals is a single complex stomach that is composed of three non-glandular compartments (the rumen, reticulum, and omasum) and one glandular compartment (the abomasum) (Budras et al., 2003). This four-compartmented stomach represents the main distinguishing characteristic of the ruminant gastrointestinal tract (Hofmann, 1988). Ruminants have the ability to digest fibrous plant material in the rumen, which contains microbial communities capable of anaerobic fermentation (NRC, 2016). The ability to utilize fiber as an energy source allows ruminants to occupy a unique niche in terrestrial ecosystems (Preston and Leng, 1987).

The rumen, reticulum, and omasum are collectively known as the forestomach and are where feed is fermented anaerobically (NRC, 2016). The reticulum is a small pouch attached to the cranial portion of the rumen and is characterized by a honeycomb-patterned epithelial lining (Pond et al., 2005; NRC, 2016). The rumen is the largest of the four stomach compartments, averaging a capacity of 202.4 L for adult cattle and 23.5 L for sheep and goats (Swenson, 1977). The rumen and reticulum together form the reticulorumen, a continuous sac that holds ingested feed, microorganisms, and gases (NRC, 2016). The reticulorumen possesses muscular grooves or pillars cranially, caudally, and longitudinally which separate the compartment into cranial, dorsal, ventral, caudodorsal, and caudoventral sacs. These pillars allow for the contraction of the reticulorumen and assist in the movement of digesta (NRC, 2016). The omasum is connected to the reticulorumen by the reticulo-omasal orifice and is characterized by numerous laminae, or sheets of tissue (Bost, 1970; Okine et al., 1998). Both the omasum and reticulorumen are lined with non-glandular stratified squamous epithelial cells (Hofmann, 1988). This tissue serves as a barrier between the forestomach and the rest of the body and is involved in the absorption and transport of metabolites (NRC, 2016). The rumen epithelium is composed of four layers of cells: the stratum corneum (closest to the lumen), the stratum granulosum, the stratum spinosum, and the stratum basale (Graham and Simmons, 2005). The luminal surface of the epithelium forms numerous small projections of epithelial tissue called papillae, which increase the surface area for absorption of the products of digestion and fermentation (Ensminger et al., 1990). The laminae of the omasum also possess small papillae to aid in absorption of water and metabolites and assist in particle reduction by grinding digesta before it enters the abomasum (Becker et al., 1963; NRC, 2016). The abomasum is connected to the omasum by the omasal-abomasal orifice and is the only stomach compartment in the ruminant that possesses digestive glands (Swenson,

1977; Ensminger et al., 1990). The abomasum functions similarly to the nonruminant stomach in that it produces mucus, pepsinogen and hydrochloric acid for the chemical digestion of feed (NRC, 2016). Digesta will then exit the gastric system and by way of the pyloric valve which leads to the intestines.

Rumen Physiology and Rumination

The digestive process is initiated in the mouth where feed is masticated to decrease particle size and is mixed with saliva (NRC, 2016). Saliva aids in the passage of the food bolus down the esophagus and provides chemical buffers to help maintain an appropriate rumen pH (Pond et al., 2005). Once swallowed, ingested feed is mixed with ruminal contents by the coordinated primary contractions of the reticulorumen (NRC, 2016). These contractions serve to aid in the process of rumination, or cud-chewing, by circulating ingesta around the reticulorumen and later propelling it on through the omasum and abomasum (Pond et al., 2005). Rumination is yet another defining feature of ruminants and involves the regurgitation of a bolus of coarse feed particles up the esophagus for remastication, reensalivation, and reswallowing (Ensminger et al., 1990; Pond et al., 2005). Remastication reduces feed particle size, which is necessary for movement of digesta out of the rumen, and the additional saliva production further assists with feed passage and rumen pH buffering (Ensminger et al., 1990). Remastication of a feed bolus generally lasts between 30 and 70 s before reswallowing occurs (NRC, 2016). Long fibrous feeds stimulate longer rumination time, where cattle on forage may spend up to 8 to 9 h/d ruminating while those on high-concentrate diets may ruminate less than 3 h/d (Welch, 1982; Beauchemin et al., 1994; Pond et al., 2005).

Eructation, or belching of gas, is a necessary action in ruminants (Pond et al., 2005). The process of fermentation in the rumen results in the formation of gases, mainly carbon dioxide and methane, which must be eliminated from the rumen to avoid bloating (Ensminger et al., 1990). Increased pressure from gas in the rumen stimulates secondary, or eructative, contractions of the rumen which occur in the caudoventral sac followed by contractions in the cranial direction (NRC, 2016). The dorsal sac of the rumen then contracts, forcing the gases into the esophagus (Pond et al., 2005). A smaller proportion of gas is not eructated but is instead absorbed by blood leaving the rumen and is carried to the lungs for exhalation (Ensminger et al., 1990).

Passage of Digesta

In ruminant animals, particularly on forage-based diets, rumen contents may be divided into a liquid phase, a buoyant raft or mat of feed particles, or small particles suspended in the liquid phase beneath the particle mat (Vieira et al., 2008). The passage of digesta from the rumen is altogether complex and involves the movement of different feed components through the gastrointestinal tract at varying rates (NRC, 2016). Soluble fractions of feed will pass with the liquid phase while large fibrous feed particles will be retained for further digestion (Mertens, 1993; NRC, 2016). In both cattle and sheep, an increase in feed intake is accompanied by an increase in digesta passage rate (Blaxter et al., 1956; Campling et al., 1961). An increase in passage rate is associated with reduced digestibility, however, especially for fibrous components (Tyrrell and Moe, 1975). Additional factors that influence passage of feed particles from the rumen are diet composition, the presence of feed additives, ambient temperature, method of feeding, and individual animal characteristics (Owens and Goetsch, 1986; NRC, 2016).

RUMEN DEGRADABILITY OF NUTRIENTS

Determination of a feed's nutrient content is an important aspect of feed analysis, but it does not offer much insight into the amount of nutrient that will become available to the animal when fed. The digestibility of a feedstuff provides an indication of the proportion that can be digested and absorbed by the animal (Pond et al., 2005; Huhtanen et al., 2006). Degradation of feedstuffs in the rumen through microbial fermentation serves to supply the ruminant animal and its rumen microbial populations with essential nutrients (Mohamed and Chaudhry, 2008; McDonald et al., 2011). Different nutrient components, however, degrade at different rates and to different extents, making rumen degradability an important aspect of feeds to investigate. Simple sugars and soluble cell wall components, for example, undergo nearly total degradation in the rumen (Van Soest, 1994), whereas ruminal starch degradability may vary between 55 and 95% as influenced by intrinsic properties of the feed and processing (Nocek and Tamminga, 1991; Offner et al., 2003; Harmon, 2009; Patton et al., 2012). Postruminal availability of the starch remainder is typically high, resulting in total-tract digestibility approaching 100% for some starchy processed feeds (Theurer et al., 1999; Firkins et al., 2001). Conversely, very little fiber digestion occurs postruminally (Moran, 2005).

Ruminal digestion of nutrients is dictated by the competing processes of the digestion rate by microbes (kd, h^{-1}) and the passage rate of feed leaving the rumen (kp, h^{-1}) (Balch, 1950; Allen and Mertens, 1988; NRC, 2016). A more rapid rate of digestion or a slower passage rate would result in increased ruminal digestion of feed while an increased passage rate or slower digestion rate would lead to decreased digestion (Dijkstra et al., 2005). The process of digestion follows the principle of mass-action and is generally described as following first-order kinetics (Mertens and Ely, 1979; Dijkstra et al., 2005). Feed (serving as the substrate) is described as

possessing both a potentially digestible fraction (PD) and an indigestible fraction (1 − PD) (Waldo et al., 1972; Dijkstra et al., 2005). The indigestible fraction may only exit the rumen by way of passage (Waldo et al., 1972; Allen and Mertens, 1988). As explained by Waldo et al. (1972) and Allen and Mertens (1988), estimated rumen digestibility of a feed may be calculated as:

$$\text{Digestibility (\%)} = kd/(kd + kp)$$

where

kd = the rate of digestion in the rumen

kp = the rate of passage out of the rumen

Obtaining the amount of feed digested in the rumen then simply involves multiplying the potentially digestible fraction by the percent digestibility (NRC, 2016):

$$\text{Feed digested} = PD \times kd/(kd + kp)$$

The rate and extent of degradation of feeds are both key parameters for estimating digestibility in the rumen (Dijkstra et al., 2005; Hackmann et al., 2010). As stated by Dijkstra and colleagues (2005), “Kinetic parameters of digestion are important because they not only describe digestion, but also they characterize the intrinsic properties of feeds that limit their availability to ruminants.” The fibrous composition of feeds influences both the rate and extent of ruminal degradation. A higher degree of fiber, and therefore lignin, would result in slower and less complete digestion (Buxton and Redfearn, 1997). As explained previously, lignin is indigestible and serves to protect the plant against degradation, thereby impeding the breakdown of other nutrient fractions (Ensminger et al., 1990; Chen, 2014). Variation between plant species as well

as degree of plant maturity may influence the fibrous content of a feed which, in turn, would contribute to differences in degradability (Kamstra et al., 1958; Buxton and Redfearn, 1997). Rumen degradability of fibrous feeds may be improved by chemical treatments containing hydroxides, peroxides, or ammonia, which partially break down lignin and hemicellulose (Kamstra et al., 1958; Fahey et al., 1993; Varga and Kolver, 1997). Processing through grinding, pelleting, or extruding, for example, may increase the digestibility of grains through exposure of the highly digestible cell contents (Rooney and Pflugfelder, 1986; Nocek and Tamminga, 1991; Murphy et al., 1994). Animal characteristics including intake and passage rate are also known to influence degradation kinetics. An increase in feed intake would result in a swifter passage rate of digesta exiting the rumen (Blaxter et al., 1956; Campling et al., 1961). This would cause more feed particles to leave the rumen before maximal digestion has occurred and represent a reduction in digestibility, particularly for structural carbohydrates (Tyrrell and Moe, 1975; Russell et al., 1992; Varga and Kolver, 1997).

Degradability of Fiber

Ruminants are generally capable of digesting around 60 to 70% of fiber from grasses (Buxton et al., 1995). A lower proportion may be digested from legumes, at approximately 40 to 50%, mainly due to a greater concentration of indigestible lignin in legume forages (Smith et al., 1972; Buxton et al., 1995). The vast majority of fiber digestion occurs in the rumen (Tamminga, 1993; Huhtanen et al., 2006). This proportion generally ranges from 80 to 100%, depending on the type and length of forage (Mertens and Ely, 1979). The proportion has been reported as greater for alfalfa compared to grasses due to the differences in NDF composition between those forage types, with grasses possessing a greater slow-digesting fraction of NDF (hemicellulose) (Smith et al., 1972; Mertens and Ely, 1979). A greater percent of long forage is digested in the

rumen compared to processed forage. This is because a reduction of forage particle size results in a swifter rate of passage out of the rumen before more extensive fiber digestion can occur (Hinders and Owen, 1968; Waldo, 1970). Ruminal fiber degradation rates for forages generally fall between 2.0 and 10%/h (Varga and Hoover, 1983; Andrighetto et al., 1993; Bhatti et al., 2008) and 5.0 and 15%/h for cereals (Varga and Hoover, 1983; Mustafa et al., 2000). A direct relationship does not appear to exist between the degradation rate of fiber and chemical composition, indicating that fiber degradation kinetics are influenced by factors beyond simple proportions of fibrous content in feeds (Smith et al., 1972; Allen and Mertens, 1988). A major factor influencing fiber degradability at the cellular level is substrate accessibility to cellulolytic bacteria, and this accessibility is determined by the chemical and physical properties of the plant's tissues (Varga and Kolver, 1997). *In vitro* studies conducted by Akin et al. (1974) and Akin and Amos (Akin and Amos, 1975) have identified differing degradation rates associated with various plant tissue types. The fastest rates of rumen bacterial degradation were associated with mesophyll and phloem tissue whereas digestion of epidermal tissue and bundle sheaths occurred more slowly (Akin and Amos, 1975). Degradation of lignified cells was not observed, even after 72 hours of incubation (Akin et al., 1974). Lignin's property as a physical barrier as well as its cross-linking with polysaccharides may contribute to impaired fiber degradation (Hoover, 1986; Iiyama et al., 1990; Jung and Allen, 1995). Besides lignin, other structural constraints to fiber degradation include plant waxes and epidermal cuticles that hinder bacteria from accessing degradable tissue (Wilson and Kennedy, 1996; Buxton and Redfearn, 1997). The epidermis of cool-season grasses such as fescue are known to degrade more quickly than that of warm-season grasses (e.g. bermudagrass), due to the latter's association with thick-walled bundle sheath cells (Buxton and Redfearn, 1997). The physical and chemical characteristics of fiber at

the cellular level have thus been demonstrated to affect digestion kinetics. Degradabilities of various feedstuffs could therefore be expected to differ, and thus result in varying nutrient supplies to the animal, even if the overall fiber concentration is similar among them.

Degradability of Starch

Around 60 to 90% of starch degradation occurs in the rumen, where starch is fermented or used in microbial matter (Ørskov, 1986; Nocek and Tamminga, 1991). Ruminal degradation rates of starch may vary widely between different types of feed. Among cereal grains, reported rates typically range from as low as 2.0%/h for sorghum to over 30%/h for wheat (Herrera-Saldana et al., 1990; Offner et al., 2003; Wang et al., 2009). Rates for forage starch generally fall between 4.0 and 12%/h, with ensiled forages occupying the higher end of the range (Offner et al., 2003; Jurjanz and Monteils, 2005; Peyrat et al., 2014). This wide variation in starch degradation kinetics may be attributed to the intrinsic properties of the particular feedstuff (Offner et al., 2003). Starch is stored by plants inside small granules where it exists in 2 forms: a helical crystalline system of amylopectin and amylose, which is dispersed throughout (Gallant et al., 1992; Knudsen, 1997; Baroux et al., 2002). These granules may be fully embedded inside a protein matrix of the endosperm, which is probably most notable in sorghum and corn grain (Rooney and Pflugfelder, 1986). As explained by French (1973), the characteristics of the starch granules, the quantities of amylose and amylopectin, and the protein matrix surrounding the starch granules are all important factors that characterize the starch content of different plant species. These factors are widely considered to be largely responsible for the variability observed in starch degradation kinetics of different feeds (Rooney and Pflugfelder, 1986; Theurer, 1986; Nocek and Tamminga, 1991; Kotarski et al., 1992; McAllister et al., 1993). The granules themselves vary in size, with granules in corn averaging around 10 to 20 μm in diameter and

tuber starch granules ranging from 20 up to 100 μm (French, 1973). A connection between increased granule size and reduced starch digestibility has been suggested, however, differences in digestibility may be due more to the granules' structural integrity than their size (Leach and Schoch, 1961; Dreher et al., 1984). Research by Leach and Schoch (1961) has demonstrated that greater granule porosity, as seen in waxy genotypes of corn and sorghum, results in easier access to and digestion by bacterial amylase compared to the less permeable granules of potato starch. Increasing the amylose fraction of a feed at the expense of amylopectin generally corresponds to a decrease in starch degradability (Rooney and Pflugfelder, 1986). This has been demonstrated in cereal grains including varieties of barley (Asare et al., 2011), sorghum (Lichtenwalner et al., 1978; Kotarski et al., 1992), and corn (Banks and Greenwood, 1975). Amylopectin is a much larger molecule than amylose and thereby provides more surface area for degradation to occur (Foster, 1965; Thorne et al., 1983). The highly branched structure of amylopectin also offers more opportunities for enzymatic attack whereas amylose is composed of single chains of glucose that are more tightly bonded together (Leach, 1965). Amylose is also known to form a complex with lipids that is resistant to degradation (Larsson and Miezis, 1979). The more important influencer of starch digestion kinetics, however, is most likely due to the protein matrix surrounding the starch granules (Kotarski et al., 1992; McAllister et al., 1993). Investigations have indicated that differences in the qualities of the protein matrix are responsible for the disparities in starch degradation rates observed between certain cereals. Under a scanning electron microscope, McAllister (1991) observed that the tough protein matrix of corn served as a protective barrier for the starch granules against entry by rumen microbes. The protein matrix associated with barley starch, however, has been demonstrated as easily and swiftly degraded by microbes (McAllister et al., 1993). Heat processing (i.e. steam-flaking)

improves the starch digestibility of grains known for slow rates of degradation, such as corn and sorghum, by disrupting the protein matrix and increasing microbial access to the endosperm interior (Theurer, 1986; Nocek and Tamminga, 1991). In the same way, ensiling of forage may increase starch digestibility over that of the fresh plant (Peyrat et al., 2014).

Degradability of Protein

Rumen microbes degrade dietary crude protein (**CP**) to obtain ammonia nitrogen and amino acids to support the synthesis of microbial protein (Tamminga, 1979; Wang et al., 2009; McDonald et al., 2011). Crude protein is broadly classified as either rumen-degradable protein (**RDP**), which is the portion that may be utilized by rumen microbes or be absorbed from the rumen as ammonia, or rumen-undegradable protein (**RUP**, or bypass protein), which escapes the rumen (DiCostanzo, 2007; McDonald et al., 2011). Microbial protein and RUP are then digested and absorbed in the small intestine (Wang et al., 2009). The process of degrading RDP is accomplished by microbial proteinases, which initially hydrolyze the peptide bonds of the protein chain and then degrade the released amino acids and peptides (Tamminga, 1979; Russell et al., 1992). Similar to the cases of other nutrients already discussed, ruminal degradation kinetics of dietary protein may also vary widely between different types of feeds (Mertens, 1977; Batajoo and Shaver, 1998). Feeds such as wheat middlings, soybeans and forages contain a greater proportion of soluble RDP protein that is swiftly degraded by microbes (Russell et al., 1992; Batajoo and Shaver, 1998; NRC, 2016). Proteins of animal origin, such as blood meal, are higher in RUP and typically display slower degradation rates for the potentially rumen-degraded protein fraction (Stern et al., 1994; Habib et al., 2013; NRC, 2016). Sorghum grain, by contrast, contains a greater fraction of RUP compared to RDP, but its proportion of potentially ruminally-degraded protein is rapidly degraded in the rumen (Wang et al., 2009; NRC, 2016). Structure and

solubility are key factors influencing the differences in degradation kinetics of proteins in various feeds. Variations in protein structure, which may be due to crosslinking or disulfide bridges, can affect rumen degradability by reducing the accessibility to proteases (Nugent and Mangan, 1978; Tamminga, 1979; Stern et al., 1994). Dietary protein solubility is affected by the proportions of glutelins and prolamins (which are less-soluble proteins) versus the proportions of albumins and globulins (which are more soluble) that it possesses (Tamminga, 1979). A feed with higher fractions of soluble proteins compared to less soluble proteins would therefore be expected to have increased nitrogen solubility over a feed with the fractions reversed (Wohlt et al., 1976; Tamminga, 1979; van Barneveld, 1999). Solubility of feed protein may also be impacted by processing (Stern et al., 1994). Processing methods involving heat (e.g. roasting, extruding) can cause protein denaturation, resulting in decreased solubility and slower ruminal degradation rate (Russell et al., 1992). Increasing the amount of bypass protein compared to RDP is beneficial in situations when intestinal digestion of high quality dietary protein is desired, such as when feeding to meet the protein requirements of growing and lactating animals (Kamalak et al., 2005). Soybean meal, for example, is a common protein source frequently heat-treated to increase its proportion of RUP, thereby protecting more of its protein from microbial enzymatic degradation and allowing it to reach the small intestine for breakdown and absorption (Stern et al., 1994).

Measuring Degradability

Estimation of nutrient digestibility kinetics in the rumen can be accomplished using *in vivo*, *in situ*, and *in vitro* methods. The *in vivo* technique involves the use of inert markers, such as chromic oxide, and sample collection from omasal or intestinal cannulae, to estimate the nutrient disappearance and flow of digesta from the rumen (Titgemeyer, 1997). The laborious,

time-consuming, and costly nature of the *in vivo* method, however, has encouraged the development of *in situ* and *in vitro* alternatives (Stern et al., 1997). With the *in situ* (or *in sacco*) method, porous nylon bags containing a ground sample of the feed of interest are placed in the rumen via a cannula and incubated to assess nutrient disappearance from the bags over time (Ørskov et al., 1980). Although it offers a fairly close alternative to the *in vivo* method by still utilizing the rumen environment, the *in situ* method is not without its drawbacks (Stern et al., 1997; Offner et al., 2003). Feed within the bags is not subjected to the particle size reduction and breakdown that would occur with normal chewing and rumination activities (Ørskov et al., 1980; Ewing and Johnson, 1987). Small feed particles may escape the bag pores undegraded, and this issue may be further affected by variation in bag pore size between different investigations (Nocek and English, 1986; Nocek, 1988; Liebe et al., 2018). Contamination of bag residues by rumen microbes (Edmunds et al., 2012) and variation in microbial activity between bag and rumen fluid can also contribute to reduced accuracy of *in situ* estimations (Nozière and Michalet-Doreau, 1996; Edmunds et al., 2012). *In vitro* procedures for estimating degradability have been utilized for feed components such as fiber (Varel et al., 1993; Fukushima et al., 2015), protein (Raab et al., 1983; Janicki and Stallings, 1988; Saleh et al., 2003), and starch (Cone et al., 1989; Michalet-Doreau et al., 1997). These methods utilize rumen fluid (Menke et al., 1979; Raab et al., 1983; Laconi and Jayanegara, 2015) or individual enzymes (de Boever et al., 1986; Janicki and Stallings, 1988; Herrera-Saldana et al., 1990) for sample incubation. Advantages of the *in vitro* method over the *in situ* and *in vivo* methods are time, labor, cost, and reduction in animal use (Edmunds et al., 2012). The main drawback is that the actual rumen environment is not used, but this may be advantageous in cases where the primary research interest is to investigate the

feed's intrinsic properties influencing degradation rather than animal effects (Varel and Kreikemeier, 1995; Dijkstra et al., 2005).

THE RUMEN MICROBIOME

The rumen microbiome is a complex ecosystem of microorganisms that include bacteria, archaea, protozoa, and fungi (Mackie et al., 2001; de Menezes et al., 2011). It forms a mutualistic symbiotic relationship with the ruminant animal by converting otherwise indigestible plant fiber into an energy source and providing nutrients in the form of microbial biomass (Hungate, 1966; Russell et al., 1992). In exchange, the ruminant host provides the microorganisms with nutrient substrates and a regulated anaerobic living environment (Schwartz and Gilchrist, 1974). The pregastric fermentative capacity of the rumen microbiome to yield energetically valuable volatile fatty acids (**VFA**) as plant fermentation end products represents the unique defining feature of ruminants (Mackie et al., 2001; Krause et al., 2013). Around 70% of the total metabolic energy utilized by the host is from these VFA, making the microbiome an important link between diet and productivity of the host animal (Bergman, 1990; Mullins et al., 2013).

Overview of Microbiome Member Characteristics

At about 10^{11} cells per g of rumen contents, bacteria account for the vast majority of microorganisms residing in the rumen and are represented by an estimated 7,000 species (Mackie et al., 2001; Chaucheyras-Durand and Ossa, 2014). Approximately 70% of the bacteria are generally found attached to feed particles whereas much of the remainder is free-floating in the rumen fluid (Preston and Leng, 1987). The major phyla found in the rumen include Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria (Chaucheyras-Durand and Ossa,

2014; Firkins and Yu, 2015). The Bacteroidetes genus *Prevotella* is typically the most abundant genus found in the rumen microbiome, presumably due to the functional diversity of its members (Griswold et al., 1999; Firkins and Yu, 2015). Collectively, the rumen bacteria produce many enzymes needed to degrade a wide range of feed substrates (Huws et al., 2018). The majority of research into the rumen microbiome has focused on the bacterial contingent compared to other microbes because bacteria represent the major players involved in substrate breakdown, fermentative activities, and microbial protein synthesis (Firkins and Yu, 2015). Despite many advances in our understanding of the rumen bacteria during the past several decades, fully characterizing aspects of their function is still a focus of current research (Huws et al., 2018).

Members belonging to the domain Archaea represent a smaller proportion of the microbiome compared to bacteria and are predominantly associated with the production of methane (Wang et al., 2017). Prominent methanogenic genera include *Methanobrevibacter* and *Methanomicrobium* (Chaucheyras-Durand and Ossa, 2014). Methane production represents an ingested energy loss for the ruminant animal in addition to contributing to greenhouse gas emissions (Kumar et al., 2013). Some methane production is necessary, however, because it rids the rumen of excess hydrogen produced during fermentation, which is required for fermentative activities to proceed (Immig, 1996).

Protozoa and fungi are the microbiome's eukaryotic representatives. Protozoa account for approximately 20% of the microbial biomass, mainly due to their larger cell volume compared to bacteria (Huws et al., 2018). Protozoa are predatory towards rumen bacteria and engulf them, which reduces rumen nitrogen efficiency through this turnover (Mackie et al., 2001; McMurphy et al., 2014). They also support methane production by providing methanogens with hydrogen (Vogels et al., 1980). For these reasons, methods to reduce protozoal populations have been

investigated as strategies to improve animal efficiency (Firkins and Yu, 2015). Fungi represent approximately 10% of the rumen microbial biomass (Krause et al., 2013). They aid in ruminal fiber degradation and are especially important when ruminants are fed high-roughage diets (Akin and Borneman, 1990; Cruywagen and van Zyl, 2008).

Coordination of Feed Degradation Activities

The degradation of feedstuffs is not accomplished by any one microorganism on its own. Rather, the various members of the rumen microbial community work together in concert to accomplish the breakdown and fermentation of complex feed substrate (Krause et al., 2013). This process is often characterized by syntropic association, in which the metabolic products yielded by one type of microbe serve as the starting substrate for another (Preston and Leng, 1987). Fungi appear to help initiate the degradation of feed particles by swiftly colonizing and disrupting lignified plant tissue with hyphae and an array of digestive enzymes (Akin and Borneman, 1990). This improves bacterial access for further breakdown. Plant polymers, including cellulose, starch, and protein, are then hydrolyzed to oligomers and smaller units by bacteria (Mackie et al., 2001). Other bacteria may then transport and ferment these products to generate VFA and compounds required by the microbes involved in degradative processes (Mackie et al., 2001; Krause et al., 2013).

The primary bacterial species responsible for fiber degradation include *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* (Cheng et al., 1991; Chesson and Forsberg, 1997). *Butyrivibrio fibrisolvens*, *Cillobacterium cellulosolvens*, and members of the *Clostridium* genus are also prominent fibrolytic species (Preston and Leng, 1987; Chesson and Forsberg, 1997). Bacterial adherence to fiber particles is accomplished through the use of

extracellular complexes called glycocalyces and cellulosomes (Weimer et al., 2006; Bensoussan et al., 2017). Cellulases and glucosidases are employed to hydrolyze cellulose to cellobiose, then to glucose (Mackie et al., 2001; McDonald et al., 2011). The more heterogeneous hemicellulose is degraded using a diverse range of enzymes into its component sugars (Krause et al., 2003).

Major starch-digesting bacteria include *Prevotella ruminicola*, *Streptococcus bovis*, *Ruminobacter amylophilus*, *Ruminococcus bromii*, *Succinimonas amylolytica*, and members of the *Bacteroides* and *Lactobacillus* genera (Kotarski et al., 1992; Cerrilla and Martínez, 2003). Starch is degraded by amylases and glucosidases to yield maltose and maltotriose and then eventually glucose (Mackie et al., 2001; Cerrilla and Martínez, 2003; McDonald et al., 2011). Rapid degradation and fermentation of starch, however, results in a decline in rumen pH which can negatively affect fiber digestion (Hoover, 1986). Fibrolytic bacterial activities become impaired as the rumen pH drops below 6 and the proliferating amylolytic populations increasingly compete for resources (Hoover, 1986).

Prominent proteolytic bacteria include *P. ruminicola*, *S. bovis*, *B. fibrisolvens*, *R. amylophilus*, and *Peptostreptococci* species (Mackie et al., 2001; McDonald et al., 2011). Bacterial proteases hydrolyze proteins into oligopeptides, then into shorter peptides and individual amino acids (Chalupa, 1976; Brock et al., 1982). Intracellularly, small peptides are hydrolyzed further and amino acids are utilized for the production of microbial protein (Tamminga, 1979). Amino acids not utilized for microbial protein synthesis are decomposed to ammonia, carbon dioxide, and carbon skeletons used to form VFA (Tamminga, 1979). The liberated ammonia nitrogen may be used for protein synthesis and microbial growth or is absorbed from the rumen into the bloodstream (McCann et al., 2014a). After being converted to urea by the liver, it may be recycled back into the rumen to again be utilized by the

microorganisms or be excreted in urine (McDonald et al., 2011; McCann et al., 2014a). The microbial protein produced in the rumen represents the majority (60-85%) of amino acids ultimately arriving in the ruminant's small intestine (Storm et al., 1983). The ability of the rumen bacteria to synthesize essential amino acids in addition to the nonessential amino acids found in microbial protein eliminates the necessity of the ruminant animal to obtain essential amino acids through the diet (McDonald et al., 2011).

Linking the Microbiome, Diet Composition, and Host Productivity

As alluded to above, rumen microbial populations show sensitivity to the availability of their preferred substrates (Gouws and Kistner, 1965). This has been frequently demonstrated in investigations into the effects of diets differing in proportions of forage and concentrate feeds (Fernando et al., 2010; de Menezes et al., 2011; Carberry et al., 2012; Petri et al., 2013; Zhang et al., 2017). Fernando and colleagues (2010) reported increased abundances of *Megasphaera elsdenii*, *S. bovis*, and *S. ruminantium* (all Firmicutes species) as well as *Prevotella bryantii* (Belonging to the Bacteroidetes Phylum) with higher levels of concentrate in the diet of beef steers, which coincided with decreases in the fiber-digesting populations of *F. succinogenes* and *B. fibrisolvens*. Their broader observations were that bacteria belonging to the phylum Fibrobacteres were more abundant in hay fed animals whereas increased numbers of Bacteroidetes were associated with high grain diets. Fernando et al. (2010) also observed that Firmicutes and Proteobacteria populations were abundant both on high forage and high concentrate diets, supporting the idea that they are core groups in the rumen microbiome possessing members with diverse substrate preferences. This helps explain why diet effects were seen more so at lower taxonomic levels compared to phylum (Fernando et al., 2010). Petri et al. (2013) reported similar findings in dairy heifers, with greater prominence of *Megasphaera*

elsdenii, *S. ruminantium*, and *Prevotella* spp. associated with a low-forage diet and increased *F. Succinogenes* associated with greater inclusion of forage. However, no difference in abundance of *S. bovis* was observed between high-grain and high-forage diets (Petri et al., 2013). Carberry et al. (2012) and Zhang et al. (2017) similarly reported lower numbers of *F. Succinogenes* on high-concentrate diets, but Carberry et al. (2012) also observed decreased *Prevotella* abundance at high grain levels, in disagreement with the findings of Petri et al. (2013), highlighting evidence of the diverse nature of this genus.

Microbial populations may be altered by varying the amounts of specific concentrate ingredients as well. Decreases in the abundance of the genus *Succinivibrio*, for instance, have been reported as distillers grains increasingly replaced a commercial grain supplement in the diet (Callaway et al., 2010). This response is further demonstration of the microbiome's sensitivity to substrate availability, as *Succinivibrio* is a starch-digesting genus and distillers grains provides little starch content compared to other concentrate feeds (Santos and Thompson, 2014; NRC, 2016). Differences in nutrient quality and availability are also likely to impact microbial abundance beyond simple forage-to-concentrate ratio or inclusion rates of specific concentrates, but this angle has not been thoroughly investigated. Work by de Menezes and colleagues (2011) has revealed increased fibrolytic bacteria in animals fed a total mixed ration (TMR) versus those consuming only pasture. Even though the pasture diet was solely forage, the greater abundance of fiber digesters associated with the TMR diet was attributed to the inclusion of straw, which would possess higher levels of lignocellulose and be less digestible than the pasture forage (de Menezes et al., 2011). Although not measured, de Menezes and colleagues (2011) suggested that the two bacterial communities observed on the different diets likely differed in terms of their metabolic potential and, ultimately, in their metabolites produced. This evidence further

underscores the importance of continued research into the influence of differences in nutrient availability on the microbiome.

Investigations have revealed links between variations in the microbiome and variations in productive ability of ruminants independent of diet. Improvements in animal efficiency offer economic benefit as well as reductions in environmental impact, which makes identifying such factors influencing productivity in ruminants an important area of study (Herrero et al., 2013). Jami and colleagues (2014) observed that distinct variations in the proportions of the two major phyla, Bacteroidetes and Firmicutes, were strongly correlated with milk fat production in dairy cows, with a greater ratio of Bacteroidetes to Firmicutes associated with decreased milk fat levels. This observation was in agreement with the findings of Indugu et al. (2017), who also reported the reverse effect for milk yield, with a higher Bacteroidetes-Firmicutes ratio seen in high-yielding cows and a lower ratio associated with lower milk yields. A proportion of this variation was attributed to the most prominent genus and Bacteroidetes member, *Prevotella*, which correlated positively with milk yield and negatively with milk fat production (Jami et al., 2014; Indugu et al., 2017). Higher abundances of *Prevotella* have also been associated with low feed efficiency in growing beef cattle (Carberry et al., 2012; McCann et al., 2014b; Myer et al., 2015). Conflicting findings exist regarding the relationship between *Prevotella* and milk production efficiency in dairy cattle, however, which could be attributed to the considerable level of diversity within the genus (Jami et al., 2014; Jewell et al., 2015; Lima et al., 2015). Lima et al. (2015) reported for example that 14 out of the 20 operational taxonomic units (OTUs) correlating positively with feed efficiency classified as *Prevotella*, but that 11 out of the 32 OTUs correlating negatively with efficiency also belonged to *Prevotella*. Species within *Prevotella* are involved in the degradation and metabolism of a wide range of feed substrates and produce a

number of different fermentation products (Carberry et al., 2012). Divergent productive activities within the genus are therefore likely to be the reason for the varied associations with host efficiency (Carberry et al., 2012; Myer, 2019).

The family *Succinivibrionaceae* has been positively correlated with beef feed efficiency (Hernandez-Sanabria et al., 2012), dairy feed efficiency (Shabat et al., 2016), and milk yield in dairy cows (Indugu et al., 2017). These effects may be due to *Succinivibrionaceae's* prominent role in propionate production which directs hydrogen away from methanogens (Denman et al., 2015; Ren et al., 2019). The glucose produced from propionate in the liver serves as an efficient energy source for the host as well as a major promoter of milk volume (Kronfeld, 1982; Hernandez-Sanabria et al., 2012). The genus *Coprococcus* has also been linked with feed efficiency (Jewell et al., 2015; Shabat et al., 2016), and likely exerts its effect through a similar mechanism as *Succinivibrionaceae* (Ungerfeld, 2015; Shabat et al., 2016). The genus *Robinsoniella*, conversely, has shown association with inefficiency and potentially acts through its production of formate, which can serve as a substrate for methane producers (Hernandez-Sanabria et al., 2012).

Total numbers of bacterial species (richness) and degree of species representation (diversity) present in the microbiome have also demonstrated linkage with production traits. Decreased species richness was reported by Lima and colleagues (2015) in high-producing dairy cows, however, efficiency of production was not measured. Shabat et al. (2016) later reported that lower degrees of richness and diversity were closely associated with higher feed efficiency. It was revealed that the more diverse microbiomes of inefficient cows utilized a broader range of metabolic pathways and produced a wide array of metabolites, whereas those of the efficient animals employed a smaller number of pathways that were more closely suited to meeting the

energetic needs of the host (Shabat et al., 2016). A smaller number of specialized, dominant taxa therefore appear to be more advantageous in terms of enhancing productive efficiency in ruminants.

Next Directions of Research

The microbial populations that have been linked to variations in ruminant host productivity likely exert their effects through the use of differing metabolic pathways (Myer, 2019). Microbiomes associated with efficient phenotypes may be characterized by a smaller number of dominant taxa that are more effective at channeling energy to the host animal and shifting fermentative products away from losses through methane (Janssen, 2010; Shabat et al., 2016). How these important bacterial populations may respond to variations in substrate availability and sources of nutrients remains a fundamental gap in our knowledge, however. As described above, a multitude of investigations have reported on microbial responses to alterations in diet forage-to-concentrate ratio, but extremely little attention has been directed towards characterization of microbial responses to changes in the availability of specific nutrients. Research is therefore required to elucidate the implications of changes to fermentative substrates on rumen bacterial populations. Such continued efforts geared toward improving our understanding of the microbiome and its interconnectedness with diet and host productivity will assist in efforts to optimize ruminant animal performance and mitigate the environmental impacts of production.

VOLATILE FATTY ACIDS

Rumen microbial fermentation of feedstuffs results in the production of short-chain volatile fatty acids (**VFA**), in addition to methane, ammonia, carbon dioxide, and small amounts

of lactic acid (Dijkstra et al., 1993; Mackie et al., 2001). The VFA are highly significant because they represent approximately 70% of the total metabolic energy utilized by the host animal (Bergman, 1990). The major VFA are acetate, propionate, and butyrate, which are 2, 3, and 4 carbons in length, respectively (Nilsson et al., 2003; Dijkstra et al., 2005). These together represent approximately 95% of the VFA produced in the rumen (Bergman, 1990). The remaining 5% of ruminal VFA are most commonly represented by valerate, isobutyrate, isovalerate, and 2-methylbutyrate. The pattern of VFA fermentation is dictated by the structure of the rumen microbiome, which is influenced by the diet fed to the host animal (Dijkstra, 1994). Feeding diets high in forage, particularly mature fibrous plant matter, results in the support of microbial populations that primarily synthesize acetate (Dijkstra et al., 2005; McDonald et al., 2011). Rumen molar proportions of acetate, propionate, and butyrate on such a diet would be expected to fall around 70:20:10 (Dijkstra et al., 2005). As the amount of nonstructural concentrates increases in the diet, amylolytic bacterial populations that produce propionate increase in prominence and the proportion of propionate increases at the expense of acetate (Ørskov, 1986; McDonald et al., 2011).

Production and Interconversion of VFA

Plant structural carbohydrates (cellulose, hemicellulose, and pectin) and nonstructural carbohydrates (mainly starch and sugars) represent the major substrate for VFA fermentation (Bergman, 1990). Degradation of these compounds yields hexoses and triose phosphate, which are metabolized to pyruvate in glycolysis (Bergman, 1990). Pyruvate may then yield acetate and butyrate using acetyl-CoA as an intermediate (Dijkstra et al., 2005). When a high-concentrate diet is fed, propionate is mainly produced from pyruvate via the acrylate pathway but, on a forage diet, propionate production via the succinate pathway increases (McDonald et al., 2011).

Dietary lipids contribute very little to VFA production, but proteins represent an important substrate source for synthesis of the minor VFA (Bergman, 1990). Following protein hydrolysis, the released amino acids are deaminated and converted: proline to valerate, valine to isobutyrate, leucine to isovalerate, and isoleucine to 2-methylbutyrate (Cotta and Hespell, 1986; McDonald et al., 2011). Although not present in the rumen in large quantities, the branched-chain minor VFA isobutyrate, isovalerate, and 2-methylbutyrate serve as essential nutrients for certain species of rumen bacteria (Cotta and Hespell, 1986). These VFA are utilized to synthesize other branched-chain compounds, including amino acids, aldehydes, and fatty acids (Allison, 1969).

Interconversions may occur between different VFA in the rumen, and this phenomenon has been explained by Ungerfeld and Kohn (2006) using thermodynamic principles. The change in Gibbs free energy (**ΔG**) for the reactions producing acetate, propionate, and butyrate from pyruvate is negative, meaning the reaction is expected to proceed spontaneously. Because the ΔG for these reactions is similar, it may be inferred that the ΔG for the VFA interconversions is near 0 and, thus, near equilibrium (Ungerfeld and Kohn, 2006). The most substantial carbon exchanges have consistently been observed between acetate and butyrate (Sharp et al., 1982; Sutton et al., 2003; Markantonatos et al., 2008). As mentioned above, acetate and butyrate are both generated from pyruvate and share acetyl-CoA as a common intermediate, a further indication of thermodynamic interconnectedness. Conversions of acetate may also be more thermodynamically probable than propionate, for instance, because it is a less reduced molecule than propionate (Ungerfeld and Kohn, 2006). Interconversions of VFA are utilized by rumen microbes to regenerate intermediates needed for particular metabolic processes (Hackmann and Firkins, 2015).

VFA Absorption and the Rumen Epithelium

Of the VFA generated in the rumen, the majority are absorbed across the rumen wall with approximately 10 to 20% passing to the omasum with rumen fluid outflow (Weston and Hogan, 1968). In addition to delivering a valuable energy source to the host animal, absorption of VFA directly from the rumen aids in maintaining the rumen's fermentative capacity by removing the products of fermentation so the reactions may continue (Gäbel et al., 2002). As explained in the above discussion on the polygastric system, the epithelium lining the rumen wall possesses many small projections of epithelial tissue called papillae, which greatly increase the surface area for absorption of VFA and other digestive products (Ensminger et al., 1990). Due to their lipid solubility, VFA are able to passively diffuse across the epithelium in their undissociated form (**HVFA**) and this process is largely determined by rumen pH (López et al., 2003). According to the Hendersson–Hasselbalch equation, a decline in pH would result in a greater proportion of HVFA and an increase in absorption rate (Gäbel et al., 2002; Dijkstra et al., 2005). This effect has been demonstrated in a number of studies (MacLeod and Ørskov, 1984; Dijkstra et al., 1993; Melo et al., 2013).

Absorption of VFA was once thought to occur exclusively via passive diffusion, but it is now known that VFA are also carried across the epithelium by way of facilitated transport mechanisms (Aschenbach et al., 2009). These transport mechanisms employ epithelial transport proteins, including monocarboxylate transporters (**MCT**) and sodium-hydrogen exchangers (**NHE**), which assist in the movement of VFA and regulation of pH (Laarman et al., 2013). The MCT function by symport, transporting a VFA anion along with a proton (Aluwong et al., 2010). Isoforms of MCT that are commonly discussed with respect to the rumen epithelium include MCT1, MCT2, and MCT4. MCT1 is expressed in the stratum spinosum and stratum basale

layers of the epithelium (Müller et al., 2002; Kirat et al., 2006). The location of MCT2 is also predominantly in the stratum spinosum and stratum basale with some expression in stratum granulosum (Kirat et al., 2013). MCT4 has been identified in all four layers of the epithelium: the stratum corneum, the stratum granulosum, the stratum spinosum, and the stratum basale (Kirat et al., 2007). The importation of protons along with VFA understandably increases the acidity of the epithelial cells, and the sodium-hydrogen exchangers assist in maintaining the cytosolic pH balance by expelling a proton back into the lumen while importing a sodium ion (Gäbel et al., 2002; Graham et al., 2007). The NHE1 and NHE3 isoforms are mainly found in the stratum granulosum, whereas NHE2 is expressed intracellularly in the stratum granulosum, stratum spinosum, and stratum basale (Graham et al., 2007).

The feeding of high-concentrate diets has long been associated with increased VFA absorption rates from the rumen compared to high-forage diets, which indicates that the epithelium can adapt its functional capacity as the fermentability of the animal's diet changes (Gäbel et al., 1991; Penner et al., 2011). This is supported by observations that greater fermentable energy intake resulted in increased MCT1 expression (Metzler-Zebeli et al., 2013) and mRNA abundance of NHE1 and NHE3 (Yang et al., 2009). Interestingly, Metzler-Zebeli et al. (2013) found that MCT4 expression actually decreased in response to increased concentrate intake, which was attributed potentially to the need to balance high VFA uptake among different modes of epithelial transport. Increased diet fermentability stimulates increased VFA production, especially through higher proportions of propionate and butyrate (Metzler-Zebeli et al., 2013). Laarman et al. (2013) observed increased abundance of MCT1 and NHE3 when butyrate was infused intraruminally in cows receiving identical high-concentrate diets, indicating that VFA profiles themselves may be the drivers of epithelial gene expression, particularly butyrate.

However, Steele et al. (2012) reported no difference in MCT1 gene expression levels despite three weeks of a high-concentrate diet treatment and elevated ruminal butyrate concentrations. These findings underscore the need for further investigation to elucidate the impacts of fermentative substrate availability and VFA profiles on epithelial transporter adaptation. An exploration of epithelial responses to dietary variations beyond simple forage-to-concentrate ratio, such as differences in ruminal availability of particular nutrient sources, would help to better explain this tissue's sensitivities to fermentative substrates and products. Additionally, investigations into linking actual rates of VFA absorption with epithelial transporter gene expression will give insight into which genes may hold the most influence over transport rates. Answers to these questions would help to inform manipulations aiming to increase the amount of energetic substrate reaching the animal's postabsorptive system.

Besides transporters, a number of other rumen epithelial genes associated with metabolism, protein regulation, and structural integrity have been investigated in an effort to gain a more holistic understanding of this complex tissue. Ones that will be discussed in this work include beta-hydroxybutyrate dehydrogenase type 1 (**BDH1**), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (**HMGCS2**), serine-threonine protein kinase (**AKT1**), heat shock protein 70 (**HSP70**), Claudin-1 (**CLDN1**), and gap-junction protein alpha 1 (**GJA1**).

BDH1 and HMGCS2 are enzymes involved in the generation of β-hydroxybutyrate (a ketone body) from butyrate, thereby creating a valuable energy substrate for various tissues (Penner et al., 2011; Naeem et al., 2012). Investigations into gene expression responses of these enzymes are generally conducted in the context of the developing rumen (Naeem et al., 2012; Liu et al., 2019), but recent work by Beckett et al. (2021) conducted with yearling heifers found that BDH1 gene expression tended to increase when beet pulp was used as a fiber source

compared to timothy hay. This expression was likely stimulated by increased levels of ruminal butyrate (Beckett et al., 2021). The same investigation could not detect differentiation in HMGCS2, however, potentially indicating differing levels of sensitivity between these genes.

AKT1 and HSP70 are involved in the management of proteins, in different capacities. AKT1 helps control protein synthesis as part of the mTOR signaling pathway and is also involved in insulin-stimulated uptake of glucose by tissues (Wang and Proud, 2006; Mackenzie and Elliott, 2014). Influences of AKT1 gene expression in the rumen epithelium are currently not fully understood, but research by Bedford et al. (2020) has suggested a tendency of AKT1 to upregulate with increased feed intake. Xue et al. (2020), however, did not observe a change in expression between normal and severely restricted intake. Further study of this important gene is therefore warranted. HSP70 is a molecular chaperone involved in a wide range of protein homeostasis activities, including the folding, transport, and degradation of proteins (Meimaridou et al., 2009). It is also expressed as part of the cellular stress response to stabilize proteins and prevent denaturation (Meimaridou et al., 2009). Downregulation of HSP70 gene expression in the rumen epithelium has been associated with decreases in ruminal pH (Hollmann et al., 2013; Beckett et al., 2021), which could indicate that protein regulation and protection may be negatively impacted under such conditions.

CLDN1 and GJA1 both have roles in maintaining the integrity of rumen epithelial tissue. CLDN1 codes for the tight-cell junction protein claudin-1, which is responsible for forming the permeability barrier in the epithelium (Graham and Simmons, 2005). GJA1 encodes connexin 43, a protein involved in the formation of gap junctions which allow communication between cells (Hsiao et al., 2010). Rumen pH depression stimulated by high-concentrate feeding has been shown to decrease expression of CLDN1 and abundance of claudin-1, which may compromise

epithelial integrity (Zhang et al., 2019). Knowledge on the influences of GJA1 expression in the mature rumen epithelium is lacking, but research in young lambs has demonstrated increased expression of GJA1 when chopped alfalfa was provided (Yang et al., 2019). This points to a sensitivity to dietary inclusion of forage, at least during rumen development.

As evidenced by the lack of data on GJA1 and other epithelial genes discussed here, rumen epithelial responses to variations in diet, VFA profiles, and rumen pH are not necessarily well understood. Considering that the rumen epithelium is responsible for delivering the rumen's energetically valuable fermentation products to the postabsorptive system, this knowledge gap needs to be overcome in order for a more complete understanding of the factors regulating the fraction of dietary energy that is ultimately available for postabsorptive metabolism. Evidence that the expression levels of epithelial genes may be altered by diet provides us with a potential avenue to manipulate the epithelium for optimal absorption of VFA. Further study is required to elucidate the effects of a wide range of diets on epithelial function as well as to provide better integration with knowledge of fermentation dynamics.

The Importance of Improving our Understanding of VFA Dynamics

Once VFA reach the animal's postabsorptive system, tissues oxidize acetate and butyrate via the krebs cycle to generate ATP while propionate is mainly utilized for gluconeogenesis (Dijkstra et al., 2005). The profile of VFA produced in the rumen may exert a significant impact on the animal's energy utilization for body function and productive activities because the VFA are utilized by the animal at different efficiencies (Thomas and Martin, 1988). Ungerfeld and Kohn (2006) explain that the production of acetate and butyrate results in the formation of CO_2 and H_2, which may then be converted to methane by rumen methanogens. Methane represents a

loss of energy because the animal cannot reclaim the H_2. Fermenting 1 mole of dietary glucose to acetate results in the generation of 1 mole of methane and butyrate formation results in 0.5 moles of methane per mole of glucose fermented (Ungerfeld and Kohn, 2006). Propionate formation, on the other hand, does not involve a net production of CO_2 and uses a net input of reducing equivalents, which diverts them from methane formation. The ratio of energy from glucose fermentation that is then retained in the fatty acid is 0.62 for acetate, 0.78 for butyrate, and 1.09 for propionate (Ungerfeld and Kohn, 2006). More energy is conserved in propionate, thereby making it more energetically useful to the animal. It is important to note that the ratio of 1.09 is referring to the ratio of energy stored in a propionate molecule versus the energy stored in a glucose molecule, as the formation of propionate does not result in the production of energy. Because VFA are not equal in terms of energetic value to the animal, being able to manipulate the rumen profile of VFA using dietary strategies would help us to optimize animal efficiency.

Past investigations into the link between diet and fermentation outcomes overwhelmingly report and discuss their VFA responses in terms of VFA concentrations. Making inferences from these studies about dietary effects on fermentation is problematic, however, because it requires the assumption that other factors, such as rumen fluid pool size and fluid passage rate, are equal across treatments. This is because influx and efflux of water will alter rumen VFA concentrations by either diluting or concentrating the solute fractions (Hall et al., 2015). Concentrations of VFA also do not relate well to VFA pool sizes or rates of production (Dijkstra et al., 1993; Hall et al., 2015). Rumen VFA concentrations should therefore not be relied upon to assess or make predictions regarding dietary effects on fermentation parameters. Discussions throughout this writing will accordingly focus on VFA dynamics rather than concentrations. Estimation of VFA dynamics may be achieved using an isotope dilution technique whereby

known amounts of ^{13}C-VFA are infused into the rumen and serial samples of rumen fluid are taken throughout the length of the infusion period (Bedford et al., 2020). Isotope enrichment of the samples is then used along with VFA concentration and fluid dynamics data to calculate estimated rates of VFA production, interconversion, and absorption. The dataset of reliable VFA dynamics estimates available in the literature is currently very small, owing mainly to the financial and labor costs associated with conducting isotope dilution experiments. This scarcity of data represents a considerable hurdle for efforts to accurately model fermentation responses to dietary variables which, in turn, limits the effectiveness of current ration formulation systems to optimize ruminant performance. Further investigations utilizing appropriate techniques to assess fermentation dynamics are therefore necessary to improve our understanding of factors that impact VFA supply.

Dietary Effects on VFA Dynamics: What We Know

Early work by Esdale and colleagues (1968) investigated ruminal acetate, propionate, and butyrate dynamics in a dairy cow fed either corn silage or alfalfa hay. They observed that daily production rates of propionate and butyrate increased on the corn silage treatment while the rate of acetate production remained the same. Accordingly, total VFA production in moles per kg of dry matter intake was higher for silage than hay. This work demonstrates that rates of propionate and butyrate production and total production of VFA increase when grain is fed. Corn silage may be classified as a forage due to its fiber content, but contains corn grain since it is made from the entire corn plant. The researchers also noted a considerable degree of interconversion between acetate and butyrate on both diets, with very little transfer associated with propionate (Esdale et al., 1968). This observation is consistent with our understanding of interconversion behavior between these three major VFA. Carbon transfer from acetate to butyrate was observed to be

greater on the corn silage treatment than on alfalfa hay (Esdale et al., 1968), indicating that VFA interconversions are impacted to some extent by diet characteristics. Sharp et al. (1982) also reported differing conversions of acetate to butyrate in steers fed whole or ground corn treatments. The same researchers observed decreased molar proportions of propionate and increased proportions of valerate when whole corn was fed. An important conclusion that can be drawn from this study is the significance of substrate availability, and that the characteristics of the nutrient source can have an impact on fermentation responses.

To study the effects of substrate type, Sutton and colleagues (2003) investigated the responses of VFA production and interconversion rates to either a "normal" diet (60% concentrate and 40% forage) or a high-concentrate diet (90% concentrate and 10% forage) fed to dairy cows. A dramatic increase in the production rate and molar proportion of propionate was observed on the high-concentrate diet compared to the normal diet, which is a logical finding given that concentrate feeds provide rapidly fermentable nonstructural carbohydrates to bacterial populations that produce propionate (McDonald et al., 2011). A slight decrease in butyrate production rate was reported in response to the high-concentrate diet but that of acetate remained similar (Sutton et al., 2003). Consistent with the investigations discussed above, Sutton et al. (2003) observed a high degree of carbon transfer between acetate and butyrate, but also noted that 13% of butyrate carbon on the normal diet and 10% on the high-concentrate diet originated from propionate. A similar study by Markantonatos et al. (2008) investigated VFA fluxes on high- and low-concentrate treatments but made differing observations. Although they similarly found a large transfer of carbon from acetate to butyrate (with greater transfer associated with the low-concentrate diet), they also determined that the contribution of propionate and butyrate to each other's pools or the acetate pool was negligible. This is in direct conflict to Sutton and

colleagues' findings of butyrate's extensive carbon exchanges with other VFA. Markantonatos and colleagues (2008) additionally reported that a significant amount of transfer occurred from acetate to propionate, which was also at odds with the observations of Sutton et al. (2003). The evidence that diet can be used to manipulate VFA dynamics as well as the conflicting reports of responses in the sparse literature underscore the continued need to study these effects.

Knowledge Gaps: The Need for Further Investigation

The cost and difficulty associated with measuring VFA dynamics has undoubtedly contributed to the fact that there exists only a very small dataset with which we may attempt to make predictions regarding fermentation responses to dietary variation. This lack of adequate data has been cited as a reason for large prediction errors in models of VFA production (Ghimire et al., 2017). The challenges with accurately modeling fermentation dynamics reduce the power of our modeling tools to fine-tune animal diets for optimal performance. Many of the past investigations also do not provide estimates or discussion on VFA absorption, tending instead to focus on production and interconversion rates. This impedes our ability to predict the proportion of VFA carried to the animal's postabsorptive system. In addition, the examination of the dynamics of minor VFA is extremely lacking. Investigations of VFA dynamics have focused virtually exclusively on acetate, propionate, and butyrate. Even though the minors may only collectively represent around 5% of the VFA present in rumen, evidence suggests that a significant degree of carbon transfer may occur from major to minor VFA and that the degree of transfer is sensitive to rumen conditions (Harthan et al., 2016). As already mentioned, minor branched-chain VFA serve as important substrates from which rumen microbes create essential amino acids for the host animal (Allison, 1969). Branched-chain VFA have also been shown to stimulate fibrolytic rumen bacteria in vitro which resulted in improved fiber digestion (Yang,

2002). These findings highlight the need to characterize these minor VFA, their relationships with major VFA, and factors influencing their behavior. A better understanding of these VFA will not only provide us with a more complete picture of rumen fermentation dynamics, but also a potential avenue to enhance microbial function and amino acid synthesis.

Another drawback of the current literature in attempting to characterize fermentation dynamics is the extremely limited amount of different diet treatments that have been used to test VFA responses. Investigations have mainly been restricted to two levels of forage-to-concentrate ratio. These provide us with important information on how fermentation may be altered with the addition of grain, but we still greatly lack understanding of how VFA dynamics are impacted over a wider range of diet conditions. This limits our ability to make predictions about how these diets influence fermentation and, ultimately, metabolizable energy supply to the animal. Valuable next steps in research would be to examine fermentation responses to differing sources and ruminal availabilities of key nutrients, such as fiber and protein. Diet characteristics are known to impact many factors that influence substrate fermentation, VFA profiles, and ultimate delivery of the VFA to the animal's postabsorptive system for metabolism. We therefore also need a better integration of dietary responses from these factors, including rumen conditions, microbial population dynamics, and epithelial function. In order to be able to characterize the ruminal process of converting feed to metabolizable energy substrates in a holistic manner, research must be conducted that examines each of these factors concurrently, which has not been accomplished to date. Because shifts in fermentation dynamics have implications for animal efficiency, gaining a clearer understanding of the factors influencing this complex process is essential for continued efforts to optimize the performance of ruminant livestock.

LITERATURE CITED

Akin, D. E., and H. E. Amos. 1975. Rumen bacterial degradation of forage cell walls investigated by electron microscopy. Appl. Microbiol. 29:692–701.

Akin, D. E., and W. S. Borneman. 1990. Role of rumen fungi in fiber degradation. J. Dairy Sci. 73:3023–3032. doi:10.3168/jds.S0022-0302(90)78989-8.

Akin, D. E., D. Burdick, and G. E. Michaels. 1974. Rumen bacterial interrelationships with plant tissue during degradation revealed by transmission electron microscopy. Appl. Microbiol. 27:1149–1156.

Allen, M. S., and D. R. Mertens. 1988. Evaluating constraints on fiber digestion by rumen microbes. J. Nutr. 118:261–270. doi:10.1093/jn/118.2.261.

Allison, M. J. 1969. Biosynthesis of amino acids by ruminal microorganisms. J. Anim. Sci. 29:797–807. doi:10.2527/jas1969.295797x.

Aluwong, T., P. I. Kobo, and A. Abdullahi. 2010. Volatile fatty acid production in ruminants and the role of monocarboxylate transporters: A review. Afr. J. Biotechnol. 9:6229–6232. doi:10.5897/AJB10.594.

Andrighetto, I., L. Bailoni, G. Cozzi, H. F. Tolosa, B. Hartman, M. Hinds, and D. Sapienza. 1993. Observations on in situ degradation of forage cell components in alfalfa and Italian ryegrass. J. Dairy Sci. 76:2624–2631.

AOAC. 2000. Official methods of analysis. 17th ed. Association of Official Analytical Chemists, Arlington, VA.

Asare, E. K., S. Jaiswal, J. Maley, M. Båga, R. Sammynaiken, B. G. Rossnagel, and R. N. Chibbar. 2011. Barley grain constituents, starch composition, and structure affect starch in vitro enzymatic hydrolysis. J. Agric. Food Chem. 59:4743–4754.

Aschenbach, J. R., S. Bilk, G. Tadesse, F. Stumpff, and G. Gäbel. 2009. Bicarbonate-dependent and bicarbonate-independent mechanisms contribute to nondiffusive uptake of acetate in the ruminal epithelium of sheep. Am. J. Physiol.-Gastrointest. Liver Physiol. 296:G1098–G1107. doi:10.1152/ajpgi.90442.2008.

Balch, C. C. 1950. Factors affecting the utilization of food by dairy cows: 1. The rate of passage of food through the digestive tract. Br. J. Nutr. 4:361–388. doi:10.1079/BJN19500060.

Banks, W., and C. T. Greenwood. 1975. Starch and its components. Halstead Press, New York, NY.

van Barneveld, S. L. 1999. Chemical and physical characteristics of grains related to variability in energy and amino acid availability in ruminants: A review. Aust. J. Agric. Res. 50:651–666.

Baroux, C., C. Spillane, and U. Grossniklaus. 2002. Evolutionary origins of the endosperm in flowering plants. Genome Biol. 3:reviews1026.1. doi:10.1186/gb-2002-3-9-reviews1026.

Batajoo, K. K., and R. D. Shaver. 1998. In situ dry matter, crude protein, and starch degradabilities of selected grains and by-product feeds. Anim. Feed Sci. Technol. 71:165–176.

Beauchemin, K. A., T. A. McAllister, Y. Dong, B. I. Farr, and K.-J. Cheng. 1994. Effects of mastication on digestion of whole cereal grains by cattle. J. Anim. Sci. 72:236–246. doi:10.2527/1994.721236x.

Becker, R. B., S. P. Marshall, and P. T. D. Arnold. 1963. Anatomy, development, and functions of the bovine omasum. J. Dairy Sci. 46:835–839. doi:10.3168/jds.S0022-0302(63)89156-0.

Beckett, L. M., C. B. Gleason, A. Bedford, D. M. Liebe, T. Yohe, M. B. Hall, K. M. Daniels, and R. R. White. 2021. Rumen volatile fatty acid molar proportions, rumen epithelial gene expression, and blood metabolite concentration responses to ruminally degradable starch and fiber supplies. J. Dairy Sci. In Press.

Bedford, A., L. Beckett, L. Harthan, C. Wang, N. Jiang, H. Schramm, L. L. Guan, K. M. Daniels, M. D. Hanigan, and R. R. White. 2020. Ruminal volatile fatty acid absorption is affected by elevated ambient temperature. Sci. Rep. 10:13092. doi:10.1038/s41598-020-69915-x.

Bensoussan, L., S. Moraïs, B. Dassa, N. Friedman, B. Henrissat, V. Lombard, E. A. Bayer, and I. Mizrahi. 2017. Broad phylogeny and functionality of cellulosomal components in the bovine rumen microbiome. Environ. Microbiol. 19:185–197. doi:10.1111/1462-2920.13561.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70:567–590. doi:10.1152/physrev.1990.70.2.567.

Bhatti, S. A., J. G. P. Bowman, J. L. Firkins, A. V. Grove, and C. W. Hunt. 2008. Effect of intake level and alfalfa substitution for grass hay on ruminal kinetics of fiber digestion and particle passage in beef cattle. J. Anim. Sci. 86:134–145.

Bidlack, J. 1992. Molecular structure and component integration of secondary cell walls in plants. In: Proceedings of the Oklahoma Academy of Science. Vol. 72. p. 51–56.

Blaxter, K. L., N. M. Graham, and F. W. Wainman. 1956. Some observations on the digestibility of food by sheep, and on related problems. Br. J. Nutr. 10:69–91. doi:10.1079/BJN19560015.

de Boever, J. L., B. G. Cottyn, F. X. Buysse, F. W. Wainman, and J. M. Vanacker. 1986. The use of an enzymatic technique to predict digestibility, metabolizable and net energy of compound feedstuffs for ruminants. Anim. Feed Sci. Technol. 14:203–214.

Bost, J. 1970. Omasal physiology. In: A. T. Phillipson, editor. Physiology of digestion and metabolism in the ruminant: Proceedings of the Third International Symposium. Oriel Press, Newcastle upon Tyne, England.

Bradstreet, R. B. 1954. Kjeldahl method for organic nitrogen. Anal. Chem. 26:185–187. doi:10.1021/ac60085a028.

Brady, G. S., and H. R. Clauser. 1991. Materials handbook. McGraw-Hill, New York, NY.

Bredon, R. M., and C. D. Juko. 1961. A semi-micro technique for crude fibre determination. J. Sci. Food Agric. 12:196–201. doi:10.1002/jsfa.2740120306.

Brock, F. M., C. W. Forsberg, and J. G. Buchanan-Smith. 1982. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. Appl. Environ. Microbiol. 44:561–569. doi:10.1128/AEM.44.3.561-569.1982.

Buxton, D. R., D. R. Mertens, K. J. Moore, L. J. Boyd, and J. E. Oldfield. 1995. Forage quality for ruminants: plant and animal considerations. Prof. Anim. Sci. 11:121–131.

Buxton, D. R., and D. D. Redfearn. 1997. Plant limitations to fiber digestion and utilization. J. Nutr. 127:814S-818S. doi:10.1093/jn/127.5.814S.

Callaway, T. R., S. E. Dowd, T. S. Edrington, R. C. Anderson, N. Krueger, N. Bauer, P. J. Kononoff, and D. J. Nisbet. 2010. Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. J. Anim. Sci. 88:3977–3983. doi:10.2527/jas.2010-2900.

Campling, R. C., M. Freer, and C. C. Balch. 1961. Factors affecting the voluntary intake of food by cows: 2. The relationship between the voluntary intake of roughages, the amount of digesta in the reticulo-rumen and the rate of disappearance of digesta from the alimentary tract. Br. J. Nutr. 15:531–540. doi:10.1079/bjn19610066.

Carberry, C. A., D. A. Kenny, S. Han, M. S. McCabe, and S. M. Waters. 2012. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. Appl. Environ. Microbiol. 78:4949–4958. doi:10.1128/AEM.07759-11.

Cerrilla, M. E. O., and G. M. Martínez. 2003. Starch digestion and glucose metabolism in the ruminant: a review. Interciencia. 28:380–386.

Chalupa, W. 1976. Degradation of amino acids by the mixed rumen microbial population. J. Anim. Sci. 43:828–834. doi:10.2527/jas1976.434828x.

Chaucheyras-Durand, F., and F. Ossa. 2014. The rumen microbiome: Composition, abundance, diversity, and new investigative tools. Prof. Anim. Sci. 30:1–12. doi:10.15232/S1080-7446(15)30076-0.

Chen, H. 2014. Biotechnology of lignocellulose: Theory and practice. Springer Netherlands, Heidelberg, Germany.

Cheng, K.-J., C. W. Forsberg, H. Minato, and J. W. Costerton. 1991. Microbial ecology and physiology of feed degradation within the rumen. In: Physiological aspects of digestion and metabolism in ruminants. Acadamic Press, Cambridge, MA. p. 595–624. Available from: https://linkinghub.elsevier.com/retrieve/pii/B978012702290150031X

Chesson, A., and C. W. Forsberg. 1997. Polysaccharide degradation by rumen microorganisms. In: The rumen microbial ecosystem. Chapman and Hall, London. p. 329–381.

Cone, J. W., W. Cliné-Theil, A. Malestein, and A. T. van ’t Klooster. 1989. Degradation of starch by incubation with rumen fluid. A comparison of different starch sources. J. Sci. Food Agric. 49:173–183. doi:10.1002/jsfa.2740490206.

Cotta, M. A., and R. B. Hespell. 1986. Protein and amino acid metabolism of rumen bacteria. In: Proceedings of 6th International Symposium on Ruminant Physiology. Prentice-Hall, Banff, Canada. p. 10–14.

Cresser, M. S., and J. W. Parsons. 1979. Sulphuric—Perchloric acid digestion of plant material for the determination of nitrogen, phosphorus, potassium, calcium and magnesium. Anal. Chim. Acta. 109:431–436. doi:10.1016/S0003-2670(01)84273-2.

Cruywagen, C. W., and W. H. van Zyl. 2008. Effects of a fungal enzyme cocktail treatment of high and low forage diets on lamb growth. Anim. Feed Sci. Technol. 145:151–158. doi:10.1016/j.anifeedsci.2007.05.038.

David, D. J. 1959. Determination of calcium in plant material by atomic-absorption spectrophotometry. Analyst. 84:536–545. doi:10.1039/AN9598400536.

Denman, S. E., G. Martinez Fernandez, T. Shinkai, M. Mitsumori, and C. S. McSweeney. 2015. Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog. Front. Microbiol. 6. doi:10.3389/fmicb.2015.01087. Available from: http://journal.frontiersin.org/Article/10.3389/fmicb.2015.01087/abstract

DiCostanzo, A. 2007. Implications of balancing feedlot diets for protein fractions (RDP and RUP) or amino acids. In: Proceedings of the 22nd Southwest Nutrition and Management Conference. University of Arizona, Tucson, AZ. p. 37–48.

Dijkstra, J. 1994. Production and absorption of volatile fatty acids in the rumen. Livest. Prod. Sci. 39:61–69. doi:10.1016/0301-6226(94)90154-6.

Dijkstra, J., H. Boer, J. Van Bruchem, M. Bruining, and S. Tamminga. 1993. Absorption of volatile fatty acids from the rumen of lactating dairy cows as influenced by volatile fatty acid concentration, pH and rumen liquid volume. Br. J. Nutr. 69:385–396. doi:10.1079/BJN19930041.

Dijkstra, J., J. M. Forbes, and J. France. 2005. Quantitative aspects of ruminant digestion and metabolism. Cabi.

Dreher, M. L., C. J. Dreher, J. W. Berry, and S. E. Fleming. 1984. Starch digestibility of foods: A nutritional perspective. Crit. Rev. Food Sci. Nutr. 20:47–71.

Edmunds, B., K.-H. Südekum, H. Spiekers, and F. J. Schwarz. 2012. Estimating ruminal crude protein degradation of forages using in situ and in vitro techniques. Anim. Feed Sci. Technol. 175:95–105.

Ensminger, M. E., J. E. Oldfield, and W. W. Heinemann. 1990. Feeds and nutrition. 2nd ed. The Ensminger Publishing Company, Clovis, CA.

Esdale, W. J., G. A. Broderick, and L. D. Satter. 1968. Measurement of ruminal volatile fatty acid production from alfalfa aay or corn silage rations using a continuous infusion isotope dilution technique. J. Dairy Sci. 51:1823–1830. doi:10.3168/jds.S0022-0302(68)87285-6.

Etheridge, R. D., G. M. Pesti, and E. H. Foster. 1998. A comparison of nitrogen values obtained utilizing the Kjeldahl nitrogen and Dumas combustion methodologies (Leco CNS 2000) on samples typical of an animal nutrition analytical laboratory. Anim. Feed Sci. Technol. 73:21–28. doi:10.1016/S0377-8401(98)00136-9.

Ewing, D. L., and D. E. Johnson. 1987. Corn particle starch digestion, passage and size reduction in beef steers: a dynamic model. J. Anim. Sci. 64:1194–1204. doi:10.2527/jas1987.6441194x.

Fahey, G. C., L. D. Bourquin, E. C. Titgemeyer, and D. G. Atwell. 1993. Postharvest treatment of fibrous feedstuffs to improve their nutritive value. In: H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, editors. Forage cell wall structure and digestibility. American Society of Agronomy, Madison, WI, USA. p. 715–766. Available from: http://doi.wiley.com/10.2134/1993.foragecellwall.c26

Fernando, S. C., H. T. Purvis, F. Z. Najar, L. O. Sukharnikov, C. R. Krehbiel, T. G. Nagaraja, B. A. Roe, and U. DeSilva. 2010. Rumen microbial population dynamics during adaptation to a high-grain diet. Appl. Environ. Microbiol. 76:7482–7490. doi:10.1128/AEM.00388-10.

Firkins, J. L., M. L. Eastridge, N. R. St-Pierre, and S. M. Noftsger. 2001. Effects of grain variability and processing on starch utilization by lactating dairy cattle. J. Anim. Sci. 79:E218–E238. doi:10.2527/jas2001.79E-SupplE218x.

Firkins, J. L., and Z. Yu. 2015. Ruminant Nutrition Symposium: How to use data on the rumen microbiome to improve our understanding of ruminant nutrition. J. Anim. Sci. 93:1450–1470. doi:10.2527/jas2014-8754.

Foster, J. F. 1965. Amylose and amylopectin properties in solution. In: Starch: Chemistry and technology. Acadamic Press, New York, NY. p. 358–376.

French, D. 1973. Chemical and physical properties of starch. J. Anim. Sci. 37:1048–1061. doi:10.2527/jas1973.3741048x.

Fukushima, R. S., M. S. Kerley, M. H. Ramos, J. H. Porter, and R. L. Kallenbach. 2015. Comparison of acetyl bromide lignin with acid detergent lignin and Klason lignin and correlation with in vitro forage degradability. Anim. Feed Sci. Technol. 201:25–37. doi:10.1016/j.anifeedsci.2014.12.007.

Gäbel, G., J. R. Aschenbach, and F. Müller. 2002. Transfer of energy substrates across the ruminal epithelium: Implications and limitations. Anim. Health Res. Rev. 3:15–30. doi:10.1079/AHRR200237.

Gäbel, G., S. Vogler, and H. Martens. 1991. Short-chain fatty acids and CO2 as regulators of Na+ and Cl- absorption in isolated sheep rumen mucosa. J. Comp. Physiol. B. 161. doi:10.1007/BF00260803. Available from: http://link.springer.com/10.1007/BF00260803

Gallant, D. J., B. Bouchet, A. Buléon, and S. Perez. 1992. Physical characteristics of starch granules and susceptibility to enzymatic degradation. Eur. J. Clin. Nutr. 46:S3–S16.

Ghimire, S., R. A. Kohn, P. Gregorini, R. R. White, and M. D. Hanigan. 2017. Representing interconversions among volatile fatty acids in the Molly cow model. J. Dairy Sci. 100:3658–3671. doi:10.3168/jds.2016-11858.

Goering, H. K., and P. J. Van Soest. 1970. Forage fiber analyses: Apparatus, reagents, procedures, and some applications. Agricultural Research Service, US Department of Agriculture.

Gorsuch, T. T. 1959. Radiochemical investigations on the recovery for analysis of trace elements in organic and biological materials. Report to the Analytical Methods Committee by the Society's First Analytical Chemistry Research Scholar. Analyst. 84:135–173. doi:10.1039/AN9598400135.

Gouws, L., and A. Kistner. 1965. Bacteria of the ovine rumen IV. Effect of change of diet on the predominant type of cellulose-digesting bacteria. J. Agric. Sci. 64:51–57. doi:10.1017/S0021859600010467.

Graham, C., I. Gatherar, I. Haslam, M. Glanville, and N. L. Simmons. 2007. Expression and localization of monocarboxylate transporters and sodium/proton exchangers in bovine rumen epithelium. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 292:R997–R1007. doi:10.1152/ajpregu.00343.2006.

Graham, C., and N. L. Simmons. 2005. Functional organization of the bovine rumen epithelium. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 288:R173–R181. doi:10.1152/ajpregu.00425.2004.

Griswold, K. E., B. A. White, and R. I. Mackie. 1999. Diversity of extracellular proteolytic activities among Prevotella species from the rumen. Curr. Microbiol. 39:187–194. doi:10.1007/s002849900443.

Habib, G., N. A. Khan, M. Ali, and M. Bezabih. 2013. In situ ruminal crude protein degradability of by-products from cereals, oilseeds and animal origin. Livest. Sci. 153:81–87.

Hackmann, T. J., and J. L. Firkins. 2015. Maximizing efficiency of rumen microbial protein production. Front. Microbiol. 6:465. doi:10.3389/fmicb.2015.00465.

Hackmann, T. J., J. D. Sampson, and J. N. Spain. 2010. Variability in in situ ruminal degradation parameters causes imprecision in estimated ruminal digestibility. J. Dairy Sci. 93:1074–1085. doi:10.3168/jds.2008-1958.

Hall, M. B. 2003. Challenges with nonfiber carbohydrate methods. J. Anim. Sci. 81:3226–3232. doi:10.2527/2003.81123226x.

Hall, M. B. 2009. Determination of starch, including maltooligosaccharides, in animal feeds: Comparison of methods and a method recommended for AOAC collaborative study. J. AOAC Int. 92:42–49. doi:10.1093/jaoac/92.1.42.

Hall, M. B. 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method: Collaborative study. J. AOAC Int. 98:397–409. doi:10.5740/jaoacint.15-012.

Hall, M. B., J. P. Jennings, B. A. Lewis, and J. B. Robertson. 2001. Evaluation of starch analysis methods for feed samples. J. Sci. Food Agric. 81:17–21. doi:10.1002/1097-0010(20010101)81:1<17::AID-JSFA758>3.0.CO;2-B.

Hall, M. B., T. D. Nennich, P. H. Doane, and G. E. Brink. 2015. Total volatile fatty acid concentrations are unreliable estimators of treatment effects on ruminal fermentation in vivo. J. Dairy Sci. S0022030215002088. doi:10.3168/jds.2014-8854.

Hansen, B. 1989. Determination of nitrogen as elementary N, an alternative to Kjeldahl. Acta Agric. Scand. 39:113–118. doi:10.1080/00015128909438504.

Harmon, D. L. 2009. Understanding starch utilization in the small intestine of cattle. Asian-Australas. J. Anim. Sci. 22:915–922. doi:10.5713/ajas.2009.r.08.

Harthan, L. B., R. R. White, and M. D. Hanigan. 2016. Effects of rumen thermodynamics on volatile fatty acid production and inteconversion in dairy cattle. In: Krakow, Poland.

Henneberg, W., and F. Stohmann. 1859. Über das Erhaltungsfutter volljährigen Rindviehs. J Landwirtsch. 3:485–551.

Hernandez-Sanabria, E., L. A. Goonewardene, Z. Wang, O. N. Durunna, and S. S. Moore. 2012. Impact of feed efficiency and diet on adaptive variations in the bacterial community in the rumen fluid of cattle. Appl. Environ. Microbiol. 78:1203–1214. doi:10.1128/AEM.05114-11.

Herrera-Saldana, R. E., J. T. Huber, and M. H. Poore. 1990. Dry matter, crude protein, and starch degradability of five cereal grains. J. Dairy Sci. 73:2386–2393. doi:10.3168/jds.S0022-0302(90)78922-9.

Herrero, M., P. Havlik, H. Valin, A. Notenbaert, M. C. Rufino, P. K. Thornton, M. Blummel, F. Weiss, D. Grace, and M. Obersteiner. 2013. Biomass use, production, feed efficiencies, and greenhouse gas emissions from global livestock systems. Proc. Natl. Acad. Sci. 110:20888–20893. doi:10.1073/pnas.1308149110.

Hinders, R. G., and F. G. Owen. 1968. Ruminal and post-ruminal digestion of alfalfa fed as pellets or long hay. J. Dairy Sci. 51:1253–1257.

Hindrichsen, I. K., M. Kreuzer, J. Madsen, and K. B. Knudsen. 2006. Fiber and lignin analysis in concentrate, forage, and feces: detergent versus enzymatic-chemical method. J. Dairy Sci. 89:2168–2176. doi:10.3168/jds.S0022-0302(06)72287-1.

Hofmann, R. R. 1988. Anatomy of the gastro-intestinal tract. In: D. C. Church, editor. The ruminant animal: Digestive physiology and nutrition. Prentice Hall, Englewood Cliffs, NJ. p. 14–43.

Hollmann, M., I. Miller, K. Hummel, S. Sabitzer, B. U. Metzler-Zebeli, E. Razzazi-Fazeli, and Q. Zebeli. 2013. Downregulation of cellular protective factors of rumen epithelium in goats fed high energy diet. J. Padmanabhan, editor. PLoS ONE. 8:e81602. doi:10.1371/journal.pone.0081602.

Hoover, W. H. 1986. Chemical factors involved in ruminal fiber digestion. J. Dairy Sci. 69:2755–2766.

Hsiao, H.-J., P.-A. Liu, H.-I. Yeh, and C.-Y. Wang. 2010. Classical swine fever virus down-regulates endothelial connexin 43 gap junctions. Arch. Virol. 155:1107–1116. doi:10.1007/s00705-010-0693-1.

Huhtanen, P., S. Ahvenjärvi, M. R. Weisbjerg, and P. Nørgaard. 2006. Digestion and passage of fibre in ruminants. In: Ruminant physiology: Digestion, metabolism and impact of nutrition on gene expression, immunology and stress. Wageningen Academic, Wageningen, the Netherlands. p. 87–138.

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

Huws, S. A., C. J. Creevey, L. B. Oyama, I. Mizrahi, S. E. Denman, M. Popova, R. Muñoz-Tamayo, E. Forano, S. M. Waters, M. Hess, I. Tapio, H. Smidt, S. J. Krizsan, D. R. Yáñez-Ruiz, A. Belanche, L. Guan, R. J. Gruninger, T. A. McAllister, C. J. Newbold, R. Roehe, R. J. Dewhurst, T. J. Snelling, M. Watson, G. Suen, E. H. Hart, A. H. Kingston-Smith, N. D. Scollan, R. M. do Prado, E. J. Pilau, H. C. Mantovani, G. T. Attwood, J. E. Edwards, N. R. McEwan, S. Morrisson, O. L. Mayorga, C. Elliott, and D. P. Morgavi. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: Past, present, and future. Front. Microbiol. 9:2161. doi:10.3389/fmicb.2018.02161.

Iiyama, K., T. B. T. Lam, and B. A. Stone. 1990. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. Phytochemistry. 29:733–737.

Immig, I. 1996. The rumen and hindgut as source of ruminant methanogenesis. Environ. Monit. Assess. 42:57–72.

Indugu, N., B. Vecchiarelli, L. D. Baker, J. D. Ferguson, J. K. P. Vanamala, and D. W. Pitta. 2017. Comparison of rumen bacterial communities in dairy herds of different production. BMC Microbiol. 17:190. doi:10.1186/s12866-017-1098-z.

Jami, E., B. A. White, and I. Mizrahi. 2014. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. PloS One. 9:e85423. doi:10.1371/journal.pone.0085423.

Janicki, F. J., and C. C. Stallings. 1988. Degradation of crude protein in forages determined by in vitro and in situ procedures. J. Dairy Sci. 71:2440–2448. doi:10.3168/jds.S0022-0302(88)79829-X.

Janssen, P. H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160:1–22. doi:10.1016/j.anifeedsci.2010.07.002.

Jewell, K. A., C. A. McCormick, C. L. Odt, P. J. Weimer, and G. Suen. 2015. Ruminal bacterial community composition in dairy cows is dynamic over the course of two lactations and correlates with feed efficiency. Appl. Environ. Microbiol. 81:4697–4710. doi:10.1128/AEM.00720-15.

Jung, H. G., and M. S. Allen. 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. J. Anim. Sci. 73:2774–2790.

Jung, H.-J. G. 1997. Analysis of forage fiber and cell walls in ruminant nutrition. J. Nutr. 127:810S-813S. doi:10.1093/jn/127.5.810S.

Jurjanz, S., and V. Monteils. 2005. Ruminal degradability of corn forages depending on the processing method employed. Anim. Res. 54:3–15.

Kamalak, A., O. Canbolat, Y. Gürbüz, and O. Özay. 2005. Protected protein and amino acids in ruminant nutrition. J. Sci. Eng. 8:84–88.

Kamstra, L. D., A. L. Moxon, and O. G. Bentley. 1958. The effect of stage of maturity and lignification on the digestion of cellulose in forage plants by rumen microorganisms in vitro. J. Anim. Sci. 17:199–208. doi:10.2527/jas1958.171199x.

Karkalas, J. 1985. An improved enzymic method for the determination of native and modified starch. J. Sci. Food Agric. 36:1019–1027. doi:10.1002/jsfa.2740361018.

Khanizadeh, S., D. Buszard, and C. G. Zarkadas. 1995. Misuse of the Kjeldahl method for estimating protein content in plant tissue. HortScience. 30:1341–1342. doi:10.21273/HORTSCI.30.7.1341.

Kirat, D., J. Masuoka, H. Hayashi, H. Iwano, H. Yokota, H. Taniyama, and S. Kato. 2006. Monocarboxylate transporter 1 (MCT1) plays a direct role in short-chain fatty acids absorption in caprine rumen. J. Physiol. 576:635–647. doi:10.1113/jphysiol.2006.115931.

Kirat, D., Y. Matsuda, N. Yamashiki, H. Hayashi, and S. Kato. 2007. Expression, cellular localization, and functional role of monocarboxylate transporter 4 (MCT4) in the gastrointestinal tract of ruminants. Gene. 391:140–149. doi:10.1016/j.gene.2006.12.020.

Kirat, D., K. I. Sallam, and S. Kato. 2013. Expression and cellular localization of monocarboxylate transporters (MCT2, MCT7, and MCT8) along the cattle gastrointestinal tract. Cell Tissue Res. 352:585–598. doi:10.1007/s00441-013-1570-5.

Kjeldahl, J. 1883. New method for the determination of nitrogen. Chem News. 48:101–102. doi:10.1007/BF01338151.

Knudsen, K. E. B. 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Anim. Feed Sci. Technol. 67:319–338. doi:10.1016/S0377-8401(97)00009-6.

Kotarski, S. F., R. D. Waniska, and K. K. Thurn. 1992. Starch hydrolysis by the ruminal microflora. J. Nutr. 122:178–190. doi:10.1093/jn/122.1.178.

Krause, D. O., S. E. Denman, R. I. Mackie, M. Morrison, A. L. Rae, G. T. Attwood, and C. S. McSweeney. 2003. Opportunities to improve fiber degradation in the rumen: Microbiology, ecology, and genomics. FEMS Microbiol. Rev. 27:663–693. doi:10.1016/S0168-6445(03)00072-X.

Krause, D. O., T. G. Nagaraja, A. D. G. Wright, and T. R. Callaway. 2013. Board-invited review: Rumen microbiology: Leading the way in microbial ecology. J. Anim. Sci. 91:331–341. doi:10.2527/jas.2012-5567.

Kronfeld, D. S. 1982. Major metabolic determinants of milk volume, mammary efficiency, and spontaneous ketosis in dairy cows. J. Dairy Sci. 65:2204–2212. doi:10.3168/jds.S0022-0302(82)82483-1.

Kumar, S., S. S. Dagar, A. K. Puniya, and R. C. Upadhyay. 2013. Changes in methane emission, rumen fermentation in response to diet and microbial interactions. Res. Vet. Sci. 94:263–268. doi:10.1016/j.rvsc.2012.09.007.

Laarman, A. H., L. Dionissopoulos, O. AlZahal, S. L. Greenwood, M. A. Steele, and B. W. McBride. 2013. Butyrate and subacute ruminal acidosis affect abundance of membrane proteins involved with proton and short chain fatty acid transport in the rumen epithelium of dairy cows. Am. J. Anim. Vet. Sci. 8:220–229. doi:10.3844/ajavsp.2013.220.229.

Laconi, E. B., and A. Jayanegara. 2015. Improving nutritional quality of cocoa pod (Theobroma cacao) through chemical and biological treatments for ruminant feeding: in vitro and in vivo evaluation. Asian-Australas. J. Anim. Sci. 28:343. doi:10.5713/ajas.13.0798.

Larsson, K., and Y. Miezis. 1979. On the possibility of dietary fiber formation by interaction in the intestine between starch and lipids. Starch-Stärke. 31:301–302. doi:10.1002/star.19790310905.

Leach, H. W. 1965. Gelatinization of starch. In: Starch: Chemistry and technology. Acadamic Press, New York, NY. p. 289–307.

Leach, H. W., and T. J. Schoch. 1961. Structure of the starch granule. Cereal Chem. 38:34–46.

Lichtenwalner, R. E., E. B. Ellis, and L. W. Rooney. 1978. Effect of incremental dosages of the waxy gene of sorghum on digestibility. J. Anim. Sci. 46:1113–1119.

Liebe, D. M., J. L. Firkins, H. Tran, P. J. Kononoff, and R. R. White. 2018. Technical note: Methodological and feed factors affecting measurement of protein A, B, and C fractions, degradation rate, and intestinal digestibility of rumen-undegraded protein. J. Dairy Sci. 101:8046–8053. doi:10.3168/jds.2018-14837.

Lima, F. S., G. Oikonomou, S. F. Lima, M. L. Bicalho, E. K. Ganda, J. C. de Oliveira Filho, G. Lorenzo, P. Trojacanec, and R. C. Bicalho. 2015. Prepartum and postpartum rumen fluid

microbiomes: Characterization and correlation with production traits in dairy cows. Appl. Environ. Microbiol. 81:1327–1337. doi:10.1128/AEM.03138-14.

Liu, L., D. Sun, S. Mao, W. Zhu, and J. Liu. 2019. Infusion of sodium butyrate promotes rumen papillae growth and enhances expression of genes related to rumen epithelial VFA uptake and metabolism in neonatal twin lambs. J. Anim. Sci. 97:909–921. doi:10.1093/jas/sky459.

Lloyd, L. E., B. E. McDonald, and E. W. Crampton. 1978. Fundamentals of nutrition. 2nd ed. W.H. Freeman and Company, San Francisco, CA.

López, S., F. D. D. Hovell, J. Dijkstra, and J. France. 2003. Effects of volatile fatty acid supply on their absorption and on water kinetics in the rumen of sheep sustained by intragastric infusions. J. Anim. Sci. 81:2609–2616. doi:10.2527/2003.81102609x.

Mackenzie, R. W., and B. T. Elliott. 2014. Akt/PKB activation and insulin signaling: a novel insulin signaling pathway in the treatment of type 2 diabetes. Diabetes Metab. Syndr. Obes. Targets Ther. 7:55–64. doi:10.2147/DMSO.S48260.

Mackie, R. I., C. S. McSweeney, and R. I. Aminov. 2001. Rumen. In: John Wiley & Sons, Ltd, editor. Encyclopedia of Life Sciences. John Wiley & Sons, Ltd, Chichester, UK. p. a0000404. Available from: http://doi.wiley.com/10.1038/npg.els.0000404

MacLeod, N. A., and E. R. Ørskov. 1984. Absorption and utilization of volatile fatty acids in ruminants. Can. J. Anim. Sci. 64:354–355. doi:10.4141/cjas84-296.

Marcó, A., R. Rubio, R. Compañó, and I. Casals. 2002. Comparison of the Kjeldahl method and a combustion method for total nitrogen determination in animal feed. Talanta. 57:1019–1026. doi:10.1016/S0039-9140(02)00136-4.

Markantonatos, X., M. H. Green, and G. A. Varga. 2008. Use of compartmental analysis to study ruminal volatile fatty acid metabolism under steady state conditions in Holstein heifers. Anim. Feed Sci. Technol. 143:70–88. doi:10.1016/j.anifeedsci.2007.05.005.

McAllister, T. A. 1991. Characterization and regulation of cereal grain digestion in ruminants [PhD Dissertation]. Univ. of Guelph, Guelph, ON, Canada.

McAllister, T. A., R. C. Phillippe, L. M. Rode, and K.-J. Cheng. 1993. Effect of the protein matrix on the digestion of cereal grains by ruminal microorganisms. J. Anim. Sci. 71:205–212. doi:10.2527/1993.711205x.

McCann, J. C., T. A. Wickersham, and J. J. Loor. 2014a. High-throughput methods redefine the rumen microbiome and its relationship with nutrition and metabolism. Bioinforma. Biol. Insights. 8:BBI-S15389. doi:10.4137/BBi.s15389.

McCann, J. C., L. M. Wiley, T. D. Forbes, F. M. Rouquette, and L. O. Tedeschi. 2014b. Relationship between the rumen microbiome and residual feed intake-efficiency of Brahman bulls stocked on bermudagrass pastures. D. Zilberstein, editor. PLoS ONE. 9:e91864. doi:10.1371/journal.pone.0091864.

McDonald, P., J. F. D. Greenhalgh, C. A. Morgan, R. Edwards, and L. Sinclair. 2011. Animal nutrition. 7th ed. Pearson, New York, NY.

McMurphy, C. P., A. J. Sexten, G. L. Mourer, E. D. Sharman, S. J. Trojan, M. J. Rincker, W. K. Coblentz, and D. L. Lalman. 2014. Effects of including saponins (Micro-Aid®) on intake, rumen fermentation and digestibility in steers fed low-quality prairie hay. Anim. Feed Sci. Technol. 190:47–58. doi:10.1016/j.anifeedsci.2014.01.007.

Meimaridou, E., S. B. Gooljar, and J. P. Chapple. 2009. From hatching to dispatching: The multiple cellular roles of the Hsp70 molecular chaperone machinery. J. Mol. Endocrinol. 42:1–9. doi:10.1677/JME-08-0116.

Melo, L. Q., S. F. Costa, F. Lopes, M. C. Guerreiro, L. E. Armentano, and M. N. Pereira. 2013. Rumen morphometrics and the effect of digesta pH and volume on volatile fatty acid absorption. J. Anim. Sci. 91:1775–1783. doi:10.2527/jas.2011-4999.

de Menezes, A. B., E. Lewis, M. O'Donovan, B. F. O'Neill, N. Clipson, and E. M. Doyle. 2011. Microbiome analysis of dairy cows fed pasture or total mixed ration diets. FEMS Microbiol. Ecol. 78:256–265. doi:10.1111/j.1574-6941.2011.01151.x.

Menke, K. H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. J. Agric. Sci. 93:217–222.

Mertens, D. R. 1977. Importance and measurement of protein insolubility in ruminant diets. In: Proc. Georgia Nutr. Conf. Vol. 30.

Mertens, D. R. 1993. Rate and extent of digestion. In: J. M. Forbes and J. France, editors. Quantitative aspects of ruminant digestion and metabolism. CAB International, Wallingford, Oxfordshire, UK. p. 13–51.

Mertens, D. R., and L. O. Ely. 1979. A dynamic model of fiber digestion and passage in the ruminant for evaluating forage quality. J. Anim. Sci. 49:1085–1095. doi:10.2527/jas1979.4941085x.

Metzler-Zebeli, B. U., M. Hollmann, S. Sabitzer, L. Podstatzky-Lichtenstein, D. Klein, and Q. Zebeli. 2013. Epithelial response to high-grain diets involves alteration in nutrient transporters and Na+/K+-ATPase mRNA expression in rumen and colon of goats. J. Anim. Sci. 91:4256–4266. doi:10.2527/jas.2012-5570.

Michalet-Doreau, B., C. Philippeau, and M. Doreau. 1997. In situ and in vitro ruminal starch degradation of untreated and formaldehyde-treated wheat and maize. Reprod Nutr Dev. 37:305–312. doi:10.1051/rnd:19970306.

Miller, E. L., A. P. Bimbo, S. M. Barlow, B. SHeridan, L. B. W. Burks, and C. B. T. B. M. B. A. B. G. H. M. B. L. B. B. E. W. G. J. H. J. J. L. L. E. O. J. P. P. S. C. S. E. S. H. Z. J. 2007. Repeatability and reproducibility of determination of the nitrogen content of fishmeal by the

combustion (Dumas) method and comparison with the Kjeldahl method: Interlaboratory study. J. AOAC Int. 90:6–20.

Mohamed, R., and A. S. Chaudhry. 2008. Methods to study degradation of ruminant feeds. Nutr. Res. Rev. 21:68–81. doi:10.1017/S0954422408960674.

Moran, J. 2005. Tropical dairy farming: Feeding management for small holder dairy farmers in the humid tropics. Csiro Publishing, Clayton, Victoria, Australia.

Müller, F., K. Huber, H. Pfannkuche, J. R. Aschenbach, G. Breves, and G. Gäbel. 2002. Transport of ketone bodies and lactate in the sheep ruminal epithelium by monocarboxylate transporter. Am. J. Physiol.-Gastrointest. Liver Physiol. 283:G1139–G1146. doi:10.1152/ajpgi.00268.2001.

Mullins, C. R., L. K. Mamedova, A. J. Carpenter, Y. Ying, M. S. Allen, I. Yoon, and B. J. Bradford. 2013. Analysis of rumen microbial populations in lactating dairy cattle fed diets varying in carbohydrate profiles and Saccharomyces cerevisiae fermentation product. J. Dairy Sci. 96:5872–5881. doi:10.3168/jds.2013-6775.

Munter, R. C., R. A. Grande, and P. C. Ahn. 1979. Analysis of animal tissue and food materials by inductively coupled plasma emission spectrometry in a university research service laboratory. ICP Inf. Newsl. 5:368.

Murphy, T. A., F. L. Fluharty, and S. C. Loerch. 1994. The influence of intake level and corn processing on digestibility and ruminal metabolism in steers fed all-concentrate diets. J. Anim. Sci. 72:1608–1615. doi:10.2527/1994.7261608x.

Mustafa, A. F., J. J. McKinnon, M. W. Ingledew, and D. A. Christensen. 2000. The nutritive value for ruminants of thin stillage and distillers' grains derived from wheat, rye, triticale and barley. J. Sci. Food Agric. 80:607–613.

Myer, P. 2019. Microbiomes in ruminant protein production and food security. CAB Rev. Perspect. Agric. Vet. Sci. Nutr. Nat. Resour. 14. doi:10.1079/PAVSNNR201914008. Available from: http://www.cabi.org/cabreviews/review/20193062865

Myer, P. R., T. P. Smith, J. E. Wells, L. A. Kuehn, and H. C. Freetly. 2015. Rumen microbiome from steers differing in feed efficiency. PloS One. 10:e0129174. doi:doi.org/10.1371/journal.pone.0129174.

Naeem, A., J. K. Drackley, J. Stamey, and J. J. Loor. 2012. Role of metabolic and cellular proliferation genes in ruminal development in response to enhanced plane of nutrition in neonatal Holstein calves. J. Dairy Sci. 95:1807–1820. doi:10.3168/jds.2011-4709.

Nilsson, N. E., K. Kotarsky, C. Owman, and B. Olde. 2003. Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. Biochem. Biophys. Res. Commun. 303:1047–1052. doi:10.1016/S0006-291X(03)00488-1.

Nocek, J. E. 1988. In situ and other methods to estimate ruminal protein and energy digestibility: a review. J. Dairy Sci. 71:2051–2069. doi:10.3168/jds.S0022-0302(88)79781-7.

Nocek, J. E., and J. E. English. 1986. In situ degradation kinetics: evaluation of rate determination procedure. J. Dairy Sci. 69:77–87. doi:10.3168/jds.S0022-0302(86)80372-1.

Nocek, J. E., and S. Tamminga. 1991. Site of digestion of starch in the gastrointestinal tract of dairy cows and its effect on milk yield and composition. J. Dairy Sci. 74:3598–3629. doi:10.3168/jds.S0022-0302(91)78552-4.

Norman, A. G. 1935. The composition of crude fibre. J. Agric. Sci. 25:529–540. doi:10.1017/S002185960001964X.

Norrish, K., and J. T. Hutton. 1977. Plant analyses by X-ray spectrometry I—Low atomic number elements, sodium to calcium. X-Ray Spectrom. 6:6–11. doi:10.1002/xrs.1300060104.

Nozière, P., and B. Michalet-Doreau. 1996. Validation of in sacco method: influence of sampling site, nylon bag or rumen contents, on fibrolytic activity of solid-associated microorganisms. Anim. Feed Sci. Technol. 57:203–210. doi:10.1016/0377-8401(95)00851-9.

NRC. 2016. Nutrient requirements of beef cattle. 8th ed. National Academies Press, Washington, DC.

Nugent, J. H. A., and J. L. Mangan. 1978. Rumen proteolysis of fraction I leaf protein, casein and bovine serum albumin. In: Proc. Nutr. Soc. Vol. 37. p. 29.

Offner, A., A. Bach, and D. Sauvant. 2003. Quantitative review of in situ starch degradation in the rumen. Anim. Feed Sci. Technol. 106:81–93. doi:10.1016/S0377-8401(03)00038-5.

Okine, E. K., G. W. Mathison, M. Kaske, J. J. Kennelly, and R. J. Christopherson. 1998. Current understanding of the role of the reticulum and reticulo-omasal orifice in the control of digesta passage from the ruminoreticulum of sheep and cattle. Can. J. Anim. Sci. 78:15–21. doi:10.2527/1991.6952177x.

Ørskov, E. R. 1986. Starch digestion and utilization in ruminants. J. Anim. Sci. 63:1624–1633.

Ørskov, E. R., F. d Hovell, and F. Mould. 1980. The use of the nylon bag technique for the evaluation of feedstuffs. Trop. Anim. Prod. 5:195–213.

Owens, F. N., and A. L. Goetsch. 1986. Digesta passage and microbial protein synthesis. In: L. P. Milligan, W. L. Grovum, and A. Dobson, editors. Control of digestion and metabolism in ruminants: Proceedings of the Sixth International Symposium on Ruminant Physiology. Prentice Hall, Englewood Cliffs, NJ. p. 196–223.

Patton, R. A., J. R. Patton, and S. E. Boucher. 2012. Defining ruminal and total-tract starch degradation for adult dairy cattle using in vivo data. J. Dairy Sci. 95:765–782. doi:10.3168/jds.2011-4183.

Peacock, T. R. 1992. Plant material preparation and determination of weight percent ash. Anal. Methods Man. Miner. Resour. Surv. Program US Dep. Inter. US Geol. Surv. 20–23. doi:10.3133/ofr92345.

Penner, G. B., M. A. Steele, J. R. Aschenbach, and B. W. McBride. 2011. Ruminant Nutrition Symposium: Molecular adaptation of ruminal epithelia to highly fermentable diets. J. Anim. Sci. 89:1108–1119. doi:10.2527/jas.2010-3378.

Petri, R. M., T. Schwaiger, G. B. Penner, K. A. Beauchemin, R. J. Forster, J. J. McKinnon, and T. A. McAllister. 2013. Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. PloS One. 8:e83424. doi:10.1371/journal.pone.0083424.

Peyrat, J., P. Nozière, A. Le Morvan, A. Férard, P.-V. Protin, and R. Baumont. 2014. Effects of ensiling maize and sample conditioning on in situ rumen degradation of dry matter, starch and fibre. Anim. Feed Sci. Technol. 196:12–21.

Pond, W. G., D. B. Church, K. R. Pond, and P. A. Schoknecht. 2005. Basic animal nutrition and feeding. 5th ed. John Wiley & Sons, Hoboken, NJ.

Porstmann, B., T. Porstmann, and E. Nugel. 1981. Comparison of chromogens for the determination of horseradish peroxidase as a marker in enzyme immunoassay. J Clin Chem Clin Biochem. 19:435–439. doi:10.1515/cclm.1981.19.7.435.

Preston, T. R., and R. A. Leng. 1987. Matching ruminant production systems with available resources in the tropics and sub-tropics. Penambul Books, Armidale, N.S.W.

Raab, L., B. Cafantaris, T. Jilg, and K. H. Menke. 1983. Rumen protein degradation and biosynthesis: 1. A new method for determination of protein degradation in rumen fluid in vitro. Br. J. Nutr. 50:569–582. doi:10.1079/bjn19830128.

Ren, H., X. Su, H. Bai, Y. Yang, H. Wang, Z. Dan, J. Lu, S. Wu, C. Cai, Y. Cao, X. Lei, and J. Yao. 2019. Specific enrichment of microbes and increased ruminal propionate production: The potential mechanism underlying the high energy efficiency of Holstein heifers fed steam-flaked corn. AMB Express. 9:209. doi:10.1186/s13568-019-0937-8.

Rhee, K. C. 2001. Determination of Total Nitrogen. Curr. Protoc. Food Anal. Chem. 00:B1.2.1-B1.2.9. doi:10.1002/0471142913.fab0102s00.

Rooney, L. W., and R. L. Pflugfelder. 1986. Factors affecting starch digestibility with special emphasis on sorghum and corn. J. Anim. Sci. 63:1607–1623. doi:10.2527/jas1986.6351607x.

Russell, J. B., J. D. O'Connor, D. G. Fox, P. J. Van Soest, and C. J. Sniffen. 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. J. Anim. Sci. 70:3551–3561. doi:10.2527/1992.70113551x.

Sáez-Plaza, P., M. J. Navas, S. Wybraniec, T. Michałowski, and A. G. Asuero. 2013. An overview of the Kjeldahl method of nitrogen determination. Part II. Sample preparation, working scale, instrumental finish, and quality control. Crit. Rev. Anal. Chem. 43:224–272. doi:10.1080/10408347.2012.751787.

Saleh, F., A. Ohtsuka, T. Tanaka, and K. Hayashi. 2003. Effect of enzymes of microbial origin on in vitro digestibilities of dry matter and crude protein in maize. J. Poult. Sci. 40:274–281.

Santos, E. de O., and F. Thompson. 2014. The family Succinivibrionaceae. In: E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, and F. Thompson, editors. The Prokaryotes: Gammaproteobacteria. Springer, Berlin, Heidelberg. p. 639–648. Available from: https://doi.org/10.1007/978-3-642-38922-1_368

Schmitter, B. M., and T. Rihs. 1989. Evaluation of a macrocombustion method for total nitrogen determination in feedstuffs. J. Agric. Food Chem. 37:992–994. doi:10.1021/jf00088a038.

Schwartz, H. M., and F. M. C. Gilchrist. 1974. Microbial interactions with the diet and the host animal. In: Digestion and Metabolism in the Ruminant. University of New England Publishing Unit, Sydney, Australia. p. 165–179.

Shabat, S. K. B., G. Sasson, A. Doron-Faigenboim, T. Durman, S. Yaacoby, M. E. Berg Miller, B. A. White, N. Shterzer, and I. Mizrahi. 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME J. 10:2958–2972. doi:10.1038/ismej.2016.62.

Sharp, W. M., R. R. Johnson, and F. N. Owens. 1982. Ruminal VFA production with steers fed whole or ground corn grain. J. Anim. Sci. 55:1505–1514. doi:10.2527/jas1982.5561505x.

Simonne, A. H., E. H. Simonne, R. R. Eitenmiller, H. A. Mills, and C. P. Cresman III. 1997. Could the Dumas method replace the Kjeldahl digestion for nitrogen and crude protein determinations in foods? J. Sci. Food Agric. 73:39–45. doi:10.1002/(SICI)1097-0010(199701)73:1<39::AID-JSFA717>3.0.CO;2-4.

Smith, L. W., H. K. Goering, and C. H. Gordon. 1972. Relationships of forage compositions with rates of cell wall digestion and indigestibility of cell walls. J. Dairy Sci. 55:1140–1147.

Steele, M. A., L. Dionissopoulos, O. AlZahal, J. Doelman, and B. W. McBride. 2012. Rumen epithelial adaptation to ruminal acidosis in lactating cattle involves the coordinated expression of insulin-like growth factor-binding proteins and a cholesterolgenic enzyme. J. Dairy Sci. 95:318–327. doi:10.3168/jds.2011-4465.

Stern, M. D., A. Bach, and S. Calsamiglia. 1997. Alternative techniques for measuring nutrient digestion in ruminants. J. Anim. Sci. 75:2256–2276.

Stern, M. D., G. A. Varga, J. H. Clark, J. L. Firkins, J. T. Huber, and D. L. Palmquist. 1994. Evaluation of chemical and physical properties of feeds that affect protein metabolism in the rumen. J. Dairy Sci. 77:2762–2786.

Storm, E., E. R. Ørskov, and R. Smart. 1983. The nutritive value of rumen micro-organisms in ruminants: 2. The apparent digestibility and net utilization of microbial N for growing lambs. Br. J. Nutr. 50:471–478. doi:10.1079/bjn19830115.

Sutton, J. D., M. S. Dhanoa, S. V. Morant, J. France, D. J. Napper, and E. Schuller. 2003. Rates of production of acetate, propionate, and butyrate in the rumen of lactating dairy cows given normal and low-roughage diets. J. Dairy Sci. 86:3620–3633. doi:10.3168/jds.S0022-0302(03)73968-X.

Sweeney, R. A., and P. R. Rexroad. 1987. Comparison of LECO FP-228 “Nitrogen Determinator” with AOAC copper catalyst Kjeldahl method for crude protein. J. AOAC Int. 70:1028–1030. doi:10.1093/jaoac/70.6.1028.

Swenson, M. J., ed. 1977. Dukes’ physiology of domestic animals. 9th ed. Cornell University Press, Ithaca, NY.

Tamminga, S. 1979. Protein degradation in the forestomachs of ruminants. J. Anim. Sci. 49:1615–1630.

Tamminga, S. 1993. Influence of feeding management on ruminant fiber digestibility. In: H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, editors. Forage cell wall structure and digestibility. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, USA. p. 571–602. Available from: http://doi.wiley.com/10.2134/1993.foragecellwall.c22

Theurer, C. B. 1986. Grain processing effects on starch utilization by ruminants. J. Anim. Sci. 63:1649–1662. doi:10.2527/jas1986.6351649x.

Theurer, C. B., J. T. Huber, A. Delgado-Elorduy, and R. Wanderley. 1999. Invited review: Summary of steam-flaking corn or sorghum grain for lactating dairy cows. J. Dairy Sci. 82:1950–1959. doi:10.3168/jds.S0022-0302(99)75431-7.

Thivend, P., C. Mercier, and A. Guilbot. 1972. Determination of starch with glucoamylase. In: General carbohydrate method. Elsevier. p. 100–105.

Thomas, P. C., and P. A. Martin. 1988. The influence of nutrient balance on milk yield and composition. In: Proceedings of the 46th University of Nottingham Easter School in Agricultural Sciences. Butterworths, London, UK. p. 97–118.

Thorne, M. J., L. U. Thompson, and D. J. Jenkins. 1983. Factors affecting starch digestibility and the glycemic response with special reference to legumes. Am. J. Clin. Nutr. 38:481–488.

Titgemeyer, E. C. 1997. Design and interpretation of nutrient digestion studies. J. Anim. Sci. 75:2235–2247.

Tyrrell, H. F., and P. W. Moe. 1975. Effect of intake on digestive efficiency. J. Dairy Sci. 58:1151–1163. doi:10.3168/jds.S0022-0302(75)84694-7.

Undersander, D. J., D. R. Mertens, and N. J. Thiex. 1993. Forage analyses procedures. Natl. Forage Test. Assoc.

Ungerfeld, E. M. 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: A meta-analysis. Front. Microbiol. 6. doi:10.3389/fmicb.2015.00037. Available from: http://journal.frontiersin.org/Article/10.3389/fmicb.2015.00037/abstract

Ungerfeld, E. M., and R. A. Kohn. 2006. The role of thermodynamics in the control of ruminal fermentation. In: K. Sejrsen, T. Hvelplund, and M. O. Nielsen, editors. Ruminant physiology:

Digestion, metabolism and impact of nutrition on gene expression, immunology and stress. Wageningen Academic, Wageningen, Netherlands. p. 55–85.

Van Soest, P. J. 1963. Use of detergents in the analysis of fibrous feeds. II. A rapid method for the determination of fiber and lignin. J Assoc Agric Chem. 46:829–835. doi:10.1093/jaoac/73.4.491.

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. 2nd ed. Cornell University Press, Ithaca, NY.

Van Soest, P. J., and R. W. McQueen. 1973. The chemistry and estimation of fibre. Proc. Nutr. Soc. 32:123–130. doi:10.1079/PNS19730029.

Van Soest, P. J., and J. B. Robertson. 1980. Systems of analysis for evaluating fibrous feeds. In: W. J. Pigden, C. C. Balch, and M. Graham, editors. Standardization of analytical methodology for feeds: proceedings. Ottawa, Ont.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597. doi:10.3168/jds.s0022-0302(91)78551-2.

Van Soest, P. J., and R. H. Wine. 1967. Use of detergents in the analysis of fibrous feeds. IV. Determination of plant cell wall constituents. J Assoc Off. Anal Chem. 50:50–55. doi:10.1093/jaoac/50.1.50.

Varel, V. H., and K. K. Kreikemeier. 1995. Comparison of in vitro and in situ digestibility methods. J. Anim. Sci. 73:578–582.

Varel, V. H., K. K. Kreikemeier, H.-J. G. Jung, and R. D. Hatfield. 1993. In vitro stimulation of forage fiber degradation by ruminal microorganisms with Aspergillus oryzae fermentation extract. Appl. Environ. Microbiol. 59:3171–3176. doi:10.1128/AEM.59.10.3171-3176.1993.

Varga, G. A., and W. H. Hoover. 1983. Rate and extent of neutral detergent fiber degradation of feedstuffs in situ. J. Dairy Sci. 66:2109–2115.

Varga, G. A., and E. S. Kolver. 1997. Microbial and animal limitations to fiber digestion and utilization. J. Nutr. 127:819S-823S. doi:10.1093/jn/127.5.819S.

Vieira, R. A. M., L. O. Tedeschi, and A. Cannas. 2008. A generalized compartmental model to estimate the fibre mass in the ruminoreticulum: 2. Integrating digestion and passage. J. Theor. Biol. 255:357–368. doi:10.1016/j.jtbi.2008.08.013.

Vogels, G. D., W. F. Hoppe, and C. K. Stumm. 1980. Association of methanogenic bacteria with rumen ciliates. Appl. Environ. Microbiol. 40:608–612. doi:10.1128/AEM.40.3.608-612.1980.

Waldo, D. R. 1970. Factors influencing the voluntary intake of forages. In: Proceedings natn. Conf. Forage Qual. Eval. Util., Univ. Nebraska, 1969. p. 22.

Waldo, D. R., L. W. Smith, and E. L. Cox. 1972. Model of cellulose disappearance from the rumen. J. Dairy Sci. 55:125–129. doi:10.3168/jds.S0022-0302(72)85442-0.

Wang, M., J. Jiang, Z. L. Tan, S. X. Tang, Z. H. Sun, and X. F. Han. 2009. In situ ruminal crude protein and starch degradation of three classes of feedstuffs in goats. J. Appl. Anim. Res. 36:23–28. doi:10.1080/09712119.2009.9707023.

Wang, S., K. Giller, M. Kreuzer, S. E. Ulbrich, U. Braun, and A. Schwarm. 2017. Contribution of ruminal fungi, archaea, protozoa, and bacteria to the methane suppression caused by oilseed supplemented diets. Front. Microbiol. 8:1864. doi:10.3389/fmicb.2017.01864.

Wang, X., and C. G. Proud. 2006. The mTOR pathway in the control of protein synthesis. Physiology. 21:362–369. doi:10.1152/physiol.00024.2006.

Weimer, P. J., N. P. J. 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. Appl. Environ. Microbiol. 72:7559–7566. doi:10.1128/AEM.01632-06.

Welch, J. G. 1982. Rumination, particle size and passage from the rumen. J. Anim. Sci. 54:885–894. doi:10.2527/jas1982.544885x.

Weston, R. H., and J. P. Hogan. 1968. The digestion of pasture plants by sheep. I. Ruminal production of volatile fatty acids by sheep offered diets of ryegrass and forage oats. Aust. J. Agric. Res. 19:419. doi:10.1071/AR9680419.

Williams, R. D., and W. H. Olmsted. 1935. A biochemical method for determining indigestible residue (crude fiber) in feces: lignin, cellulose, and non-water-soluble hemicelluloses. J Biol Chem. 108:653–666. doi:10.1007/s13197-011-0365-5.

Wilson, J. R., and P. M. Kennedy. 1996. Plant and animal constraints to voluntary feed intake associated with fibre characteristics and particle breakdown and passage in ruminants. Aust. J. Agric. Res. 47:199–225.

Wohlt, J. E., C. J. Sniffen, W. H. Hoover, L. L. Johnson, and C. K. Walker. 1976. Nitrogen metabolism in wethers as affected by dietary protein solubility and amino acid profile. J. Anim. Sci. 42:1280–1289.

Xue, Y., L. Lin, F. Hu, W. Zhu, and S. Mao. 2020. Disruption of ruminal homeostasis by malnutrition involved in systemic ruminal microbiota-host interactions in a pregnant sheep model. Microbiome. 8:138. doi:10.1186/s40168-020-00916-8.

Yang, B., H. Chen, J. Cao, B. He, S. Wang, Y. Luo, and J. Wang. 2019. Transcriptome analysis reveals that alfalfa promotes rumen development through enhanced metabolic processes and calcium transduction in Hu lambs. Front. Genet. 10:929. doi:10.3389/fgene.2019.00929.

Yang, C.-M. J. 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. doi:10.3168/jds.S0022-0302(02)74181-7.

Yang, W., H. Martens, and Z. Shen. 2009. Effects of energy intake and ruminal SCFA on mRNA expression of Na+/H+ exchangers in rumen epithelium of goats. In: Proc. 11th Int. Symp. of Rumin. Physiol., Clermont-Ferrand, France. p. 412–413.

Zarcinas, B. A., B. Cartwright, and L. R. Spouncer. 1987. Nitric acid digestion and multi-element analysis of plant material by inductively coupled plasma spectrometry. Commun Soil Sci Plant Anal. 18:131–146. doi:10.1080/00103628709367806.

Zhang, J., H. Shi, Y. Wang, S. Li, Z. Cao, S. Ji, Y. He, and H. Zhang. 2017. Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in Holstein heifers. Front. Microbiol. 8:2206. doi:10.3389/fmicb.2017.02206.

Zhang, K., M. Meng, L. Gao, Y. Tu, and Y. Bai. 2019. Rumen-derived lipopolysaccharide induced ruminal epithelium barrier damage in goats fed a high-concentrate diet. Microb. Pathog. 131:81–86. doi:10.1016/j.micpath.2019.02.007.

CHAPTER 3: Evaluating the Relationship Between *In Vitro* and *In Situ* Starch Degradation Rates

This chapter will be published as: Gleason, C. B., L. M. Beckett, B. R. dos Reis, and R. R. White. SHORT COMMUNICATION: Evaluating the relationship between *in vitro* and *in situ* starch degradation rates. Animal Feed Science and Technology.

ABSTRACT

Determining starch degradability is important because of its implicit relation to gut health, energy availability, feed intake, and animal productivity. *In vitro* assays for protein and fiber degradability have been useful tools for ration formulation; however, analogous assessments of starch degradability have limited updates. Our objective was to test how an enzymatic *in vitro* starch degradation assay compared with the *in situ* technique in terms of its ability to rank feeds based on their starch degradation rates. Samples of 19 livestock feeds were obtained from 4 feed mills and subjected to *in vitro* and *in situ* starch degradation analyses. Seven ruminally cannulated wethers (Suffolk, Dorset or Suffolk × Dorset) were used for the *in situ* procedure. *In vitro* starch degradation was assessed using an acetate buffer technique with modified enzymatic incubation times: amylase (**AML**) 5 min and amyloglucosidase (**AMG**) 10 min; AML 10 min and AMG 20 min; AML 15 min and AMG 30 min; AML 30 min and AMG 60 min; AML 45 min and AMG 90 min; and AML 60 min and AMG 120 min. *In situ* rates were determined using the Ørskov method and *in vitro* predicted rates calculated assuming exponential decay. Relationships were analyzed as a linear mixed-effects model with *in situ* degradation rate as the response variable and *in vitro* predicted degradation rate, and initial free glucose, starch, crude protein, neutral detergent fiber (**NDF**), and fat content as fixed effects. Interactions between *in vitro* predicted degradation rate and the nutrient percentages were also included. Feed source was included as a random effect. *In vitro* predicted starch degradation rates ranged from 2.98 to 279 %/h and *in situ* degradation rates ranged from 1.53 to 42.8 %/h. A relationship between *in vitro* and *in situ* methods was observed when starch, fat, NDF, and initial free glucose contents were accounted for. Interactions between *in vitro* predicted degradation rate and starch and NDF contents were also observed, suggesting that these nutrients may

confound the relationship between *in vitro* and *in situ* starch degradation measurements. Removing the rapidly degradable starch fraction from *in situ* analysis calculations improved the strength of the relationship between *in situ* and *in vitro* starch degradation rate. This *in vitro* assay may prove more useful for determining relative rankings of starch degradation rates among different feedstuffs rather than serving as an alternative to the *in situ* method. Additional investigation into assay repeatability and comparisons with true *in vivo* estimations of starch degradation are needed to confirm the usefulness of this *in vitro* screening technique.

Keywords: *in situ* incubation, *in vitro* assay, ruminal starch degradability

INTRODUCTION

Starch often represents a major fermentative fraction of ruminant livestock diets and is known to positively impact animal performance at optimal concentrations (Whiting, 1957; Grant, 2005; Boerman et al., 2015). Determination of starch concentration is typically conducted by enzymatic-colorimetric assay in which a sample undergoes enzymatic incubations with α-amylase and amyloglucosidase in an acetate buffer solution, followed by quantification of released glucose using glucose oxidase-peroxidase reagent to quantify glucose (Hall, 2015). Ruminal degradability of starch may vary widely between feed types, making estimation of starch degradation rates an important component of feed evaluation (Offner et al., 2003). Therefore, building upon the current database of starch degradation of feeds would be expected to help improve the understanding of feed nutritive value and enhance ration formulation systems in livestock production settings (Wang et al., 2009).

Ruminal degradation rates of various feed components, including starch, are often evaluated by the *in situ* method where nylon bags containing the feed sample are incubated in a cannulated ruminant (Ørskov and McDonald, 1979; Bruno-Soares et al., 2000; Wang et al., 2009). However, the growing encouragement to seek non-animal alternatives in research underscores the importance of developing dependable *in vitro* methods to determine nutrient degradability. *In vitro* assays for determining crude protein (**CP**) and fiber degradability have proven useful for investigations comparing different feedstuffs and subsequent nutritional value to livestock (Raab et al., 1983; Janicki and Stallings, 1988; Varel et al., 1993; Fukushima et al., 2015; Laconi and Jayanegara, 2015). Such assays intended for determination of starch degradation have not been as frequently utilized or updated. Even though ruminal starch degradation kinetics are less important when evaluating contributions to the animal's energy

supply (because a considerable portion of the starch remainder may become available postruminally (Harmon, 2009)), they become very relevant when considering rumen health (Zebeli et al., 2010) and precision feeding concepts such as nutrient synchrony (Hall and Huntington, 2008; Hersom, 2008). An *in vitro* assay utilizing similar starch-degrading enzymes as the rumen bacteria may also provide the opportunity for further study of amylolytic activities in the degradation of feedstuffs. Therefore, the objectives of this study were to (1) to assess starch degradation of a variety of common feedstuffs using an *in vitro* technique based on a starch analysis method (Hall, 2015) and (2) to compare the *in vitro* predicted results to starch degradation rates estimated by the *in situ* rumen incubation method. We hypothesized that a relationship exists between *in vitro* predicted starch degradation rates and rates estimated by the *in situ* method. The findings of this work carry potential benefit for the field of ruminant nutrition as they may enhance our understanding of starch degradation and the relationship to non-animal techniques. A better understanding of the relationship between *in vitro* and *in situ* methods, and how to account for potential differences, would also be valuable from the standpoints of both animal welfare and research expense because it would help to reduce the need for animals as experimental subjects in future studies as well as increase the speed of analysis.

MATERIALS AND METHODS

Animals, Housing, and Sample Assignments

All animal use and procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (Protocol #19-074). Seven ruminally cannulated commercial wethers (Suffolk, Dorset or Suffolk × Dorset) were individually stalled at the Smithfield Farm, Virginia

Tech, Blacksburg, VA. Wethers were approximately 2 years of age and weighed an average of 70 kg at the beginning of the experiment. Timothy hay (916 g/kg dry matter) and fresh, clean water were available *ad libitum*. The 19 feed samples that were evaluated were obtained from 4 different mills and represented a wide range of common feeds used in livestock rations. Samples were randomly assigned to a wether for *in situ* incubation. Replications across wethers were not performed.

In Situ *Determination of Starch Degradation*

Samples were dried for 24 h at 55°C in a forced-air oven and ground to pass through a 1 mm screen of a Wiley mill (Thomas Scientific, Swedesboro, NJ). Five grams of ground sample were placed in *in situ* bags (Ankom R1020, 10 × 20 cm and 50 ± 10 micron porosity, Ankom Technology, Macedon, NY) for a total of 40 bags per sample which were then heat-sealed. A 30.5 × 40.6 cm mesh bag (mDesign 01963MDL, MetroDecor, San Diego, CA) was placed in the rumen to contain the *in situ* bags. Four *in situ* bags were soaked in warm water (approximately 39°C) for 15 min then added to the mesh bag at -96 h, -48 h, -24 h, -12 h, -6 h, -3 h, -1.5 h, -1 h, and -0.5 h relative to their removal. At 0 h, all *in situ* bags were removed and gently hand washed in cool water along with the last set of 4 soaked unincubated bags. Bags were then dried for 48 h at 55°C and weighed to assess dry matter loss. The contents were pooled by time point for each sample (for a total of 10 time points) and subjected to the acetate buffer method of starch analysis, which measures starch content based on the glucose liberated from incubations with the enzymes amylase and amyloglucosidase (Hall, 2015). The amount of starch remaining at each time point was calculated by multiplying the starch concentration by the mean dry matter content of the time point's 4 *in situ* bags. Starch degradability was determined using the equation of Ørskov and McDonald (1979):

$p = a + b\ (1 - e^{-kt})$,

where p represents nutrient disappearance (%), a is the rapidly degradable fraction, b is the potentially degradable fraction, k is the rate of degradation of b, e is the base of the natural logarithm, and t is time in hours. The undegraded fraction of the sample is then calculated as

$100 - (a + b)$.

A second set of degradation rates were calculated based on removal of the starch loss measured in the unincubated *in situ* bags (the a fraction). To generate this set, the amount of starch disappearance in the unincubated bags was subtracted from the starch disappearance measured at the other time points and the equation

$p = b\ (1 - e^{-kt})$

was then used to obtain starch degradation rates adjusted for initial starch disappearance.

In Vitro *Determination of Starch Degradation*

In vitro measurement of starch degradation was carried out using a modified version of an acetate buffer method of starch determination (Hall, 2015), where enzymatic incubation times were incrementally adjusted (Table 1) in order to assess the amount of starch degradation occurring at varying time points so that the rate of degradation could be calculated. Briefly, 0.20 g of ground sample was added to a 25 × 200 mm glass tube and an additional tube contained 0.20 g of α-D-glucose (CAS: 50-99-7, VWR, Solon, OH) as a standard. Heat-stable α-amylase (Thermostable Amylase HTL, BIO-CAT, Troy, VA, Enzyme Commission number: 3.2.1.1) (0.1 mL) and 30 ml 0.1 M acetate buffer (pH 5.0) were then added and the tubes vortexed. Tubes were incubated at 100°C for the required length of time (Table 1). Amyloglucosidase (E-

AMGDF-100ML, 3,260 U/mL, Megazyme, Bray, Ireland, Enzyme Commission number: 3.2.1.3) (200 U) was added to each sample and incubated at 50°C (Table 1). Distilled water (20 mL) was then added to each tube and 1 mL of each sample solution was transferred to a microfuge tube and centrifuged at 12,000 × g for 10 min. To create the standard curve, the glucose sample solution was diluted with distilled water to 90, 80, 70, 60, 50, 40, 30, and 20% of its original volume. An amount of 6.45 µL was pipetted from each sample solution or standard solution onto a flat-bottomed 96-well plate in triplicate. Glucose oxidase-peroxidase reagent (194 µL) was then mixed into each well. The plate was covered with Parafilm (Bemis Company Inc, Neenah, WI), incubated in a 50°C oven for 20 min, and the absorbances read on a spectrophotometer (Epoch2 Microplate Reader, Biotek, Winooski, VT) at 505 nm. Starch content of the samples were obtained from the absorbances using the linear and quadratic coefficients of the standard curve as follows:

$$\text{Starch, \%} = \text{intercept} + \text{abs} \times \text{linear coefficient} + \text{abs}^2 \times \text{quadratic coefficient}$$

where *abs* equals the blank well corrected absorbance of the sample. Percentages were then corrected for volume of fluid used in the analysis and sample dry matter content. *In vitro* predicted rates of degradation were calculated using the exponential decay formula

$$p = a + (1 - e^{-kt})$$

where p represents nutrient disappearance (%), a is the rapidly degradable fraction, k is the degradation rate, and t is time. Initial free glucose content of feeds was determined by subjecting the samples to the full-length *in vitro* starch determination assay without the addition of α-amylase or amyloglucosidase.

Statistical Analysis

All data were analyzed using R Statistical Software v3.6.1 (R Core Team, 2019) and model derivation was conducted using the lme4 package (Bates et al., 2015). Relationships were analyzed using a linear mixed-effects model with either *in situ* degradation rate or *in situ* degradation rate adjusted for 0 h starch disappearance as the response variable. Fixed effects included *in vitro* predicted degradation rate, and initial free glucose, starch, CP, NDF, and fat content. Interactions between *in vitro* predicted degradation rate and starch, CP, NDF, and fat content were also tested. Feed source (i.e. mill) was included as a random effect. ANOVA was performed on the model and significance level was set at $P < 0.05$ and a tendency considered when $0.05 \leq P < 0.10$. Model concordance with measurements (i.e., agreement among measurement methods, adjusted for covariates) was assessed on the basis of concordance correlation coefficient (**CCC**).

RESULTS AND DISCUSSION

Starch degradation rates of the feed samples determined by *in vitro* and *in situ* incubation are presented in Table 2. Significant variables for the prediction of *in situ* starch degradation rate are given in Table 3. *In vitro* predicted starch degradation rates ranged from 2.98 to 279 %/h and *in situ* degradation rates ranged from 1.53 to 42.8 %/h (Table 2). The differences in ranges between the techniques were not unexpected because the measurements obtained *in vitro* were not meant to be interpreted in the same biological manner as the *in situ* measurements, but may be relevant with adjustment in the appropriate context.

In Vitro *Starch Degradation*

In an *in vitro* ranking of samples from fastest to slowest predicted starch degradation, the barley, oat hull, and wheat middling samples degraded most rapidly and were followed by the cornmeal, pea, and steam flaked corn samples. The other samples in descending order were the canola meals, soy hull #2, dried distillers grains with solubles (**DDGS**) #2, alfalfa, soy hull #1, beet pulp, and DDGS #1. The same feed sample types (e.g., soy hulls, DDGS) that did not rank consecutively were each from different feed mill sources, so differences in feed characteristics offer a possible explanation for the lack in continuity. Upon visual inspection of both DDGS samples in their underground states, for example, #1 was noticeably darker in color and had a much coarser consistency than #2. These differences in appearance may indicate that relevant differences in characteristics exist between the two sources of DDGS that may have contributed to different rates between *in situ* starch degradation estimations and starch degradation rates predicted *in vitro*. Heat damage may have been a contributing factor, but was not assessed.

Few publications exist that compare *in vitro* starch degradation rates on more than 2 feeds, making extensive comparisons of our results with the literature difficult. Nevertheless, an amyloglucosidase incubation procedure carried out by Herrera-Saldana and colleagues (1990) ranked oats, then barley, then corn in terms of most rapid starch degradation in agreement with our findings. In addition, Michalet-Doreau et al. (1997) observed faster starch degradation for wheat than corn, as indicated by amounts of *in vitro* gas production. Barley was shown to have a slightly faster rate of starch degradation than wheat when incubated in rumen fluid followed by incubation with amyloglucosidase (Sveinbjörnsson et al., 2007). Our observations are consistent with these reports.

In Situ *Starch Degradation*

The #1 pea sample and wheat middling samples had the fastest starch degradation when determined by the *in situ* method. The rest of the samples in descending order were cornmeal #2, DDGS #1, barley #2, beet pulp, canola meal #1, peas #2, steam flaked corn #1, DDGS #2, soyhull #1, oat hulls, canola meal #2, steam flaked corn #2, alfalfa, soyhull #2, barley #1, and cornmeal #1. Overall, this ranking varied from the ranking determined using the *in vitro* technique. Both methods identified the wheat middling samples as possessing faster rates of starch degradation, the pea #2 and steam flaked corn #1 samples as moderate, and the alfalfa and soyhull #2 samples as having slower rates of starch degradation relative to the collection of feeds tested. The remainder of the samples were ranked differently between the two methods, indicating that the results were being impacted by method-specific factors.

Previous reports of *in situ* starch degradation rates have typically only examined a few very prominent whole cereal grains such as corn, wheat, and barley, making extensive comparisons of literature results with our wide range of feed samples difficult. Nevertheless, the starch degradation rate of our #2 pea sample (8.63%/h; Table 2) was consistent with that reported by Wang and colleagues (2009). The starch content of the other pea sample, however, declined much more rapidly (42.8%/h; Table 2). Similarly, we observed a starch degradation rate for the #1 cornmeal sample consistent with the findings of Wang et al. (2009) but that of the #2 cornmeal sample was higher by comparison and closer to the report of Herrera-Saldana et al. (1990). Starch degradation rates of both barley samples were lower than previous reports (Herrera-Saldana et al., 1990; Wang et al., 2009). These inconsistencies may be due to between-study variation or variation between different feed samples.

In Vitro-In Situ *Comparison and the Effect of Nutrient Content*

Linear regression revealed a significant relationship ($P = 0.0092$; Table 3) between *in vitro* and *in situ* starch degradation rates of the feeds tested. The concordance correlation coefficient of the model was 0.85, indicating modest model quality. Eliminating the rapidly degradable starch fraction from *in situ* analysis data calculations improved model quality substantially (CCC = 0.98). This suggests that the instantaneously available "washout" fraction of starch may contribute to variation between estimations of *in vitro* and *in situ* starch degradation rates in the current study. Interpretations of this "washout" fraction are expected to be variable, however, because it is essentially a function of the difference between feed particle size and bag pore size (Seifried et al., 2015).

Previous studies also report agreement between *in vitro* and *in situ* measurements of starch degradation (Herrera-Saldana et al., 1990; Michalet-Doreau et al., 1997). These studies differed from ours, however, in that Michalet-Doreau et al. (1997) based their *in vitro* estimations on gas production and both used fistulated cattle rather than sheep as described here. In our model, initial free glucose, starch, fat, and NDF content along with the interaction of *in vitro* starch degradation rate and starch and NDF contents were also significant predictors for *in situ* starch degradation (Table 3). These interactions suggest that the nutrient composition of the feed influences the *in vitro* and *in situ* assays differently. Accounting for differences in composition was therefore essential to compare among starch degradation rates defined by each. As explained by French (1973), major factors characterizing starch in different plants include starch granule size and shape, content of amylose and amylopectin, and the surrounding protein matrix. Starch digestibility in the rumen is known to be impacted by the characteristics of surrounding materials, protein matrix, and cell wall features (Kotarski et al., 1992). The

appearance of significant effects of nutrient components in our prediction of *in situ* starch degradation is therefore unsurprising. It has been suggested that variation in starch digestion kinetics may be at least partially controlled by differences in the degradation rate of the protein matrix surrounding the starch granules (Theurer, 1986; McAllister et al., 1993) and such effects of the protein matrix on starch degradation have been demonstrated in a gravimetric *in vitro* system utilizing rumen fluid (McAllister et al., 1993). Herrera-Saldana et al. (1990) attributed slow initial degradation of wheat to this phenomenon. Herrera-Saldana and colleagues (1990) also noted that as the amount of fiber in a feed sample increased, the standard deviation and coefficient of variation of the feed's starch percentage tended to increase as well. The significance of fat content in our model for prediction of *in situ* starch degradation is also logical because the amylose fraction of starch is known to form a complex with lipids that exhibits resistance to enzymatic degradation (Panyoo and Emmambux, 2017). Larsson and Miezis (1979) demonstrated decreased degradation of potato starch by α-amylase when complexed with lipids and suggested this effect of lipid content as a potential source of error in enzymatic determinations of starch. Because our *in vitro* assay only utilizes enzymes that target starch granules, it is reasonable that the assay would operate differently than the rumen environment, where the suite of microbial enzymes is able to attack the various nutrient components in sequence.

Potential Challenges with* In Vitro-In Situ *Comparisons

The *in situ* technique is currently the most widely used method to estimate the digestion rate of starch (Huhtanen and Sveinbjörnsson, 2006). Its major advantage is that it utilizes the actual rumen environment (Sveinbjörnsson et al., 2007), the rumen being the major site of cereal grain starch digestion (Theurer, 1986). However, the low repeatability and the lack of

reproducibility of this technique have been criticized and considerable between-laboratory variation has been reported (Offner et al., 2003). Potential sources of variation in degradability measurements that have been identified include the loss of undegraded starch granules from nylon bags (Nocek, 1988), varying bag pore sizes (Nocek and English, 1986), the absence of within-bag particle size reduction (Ewing and Johnson, 1987), and varied activity of the rumen microbes (Nocek, 1988; Nozière and Michalet-Doreau, 1996). The CCC of our model relating *in vitro* to conventionally derived *in situ* degradation rates of starch indicates that there still exists a portion of variation unexplained by this relationship. Some of the sources of inconsistencies in *in situ* outcomes may be responsible for a portion of this variation, as indicated by model improvement when the rapidly degradable starch fraction was removed from the *in situ* analysis. Another potential challenge with our comparison is that the enzymatic profile in a typical rumen is very different compared to the enzymes we utilized *in vitro*, which were α-amylase and amyloglucosidase. Enzymes produced by the rumen microorganisms that are known to be involved with starch digestion include α-amylase, β-amylase, iso-amylase, pullulanase, and α-limit dextrinase (Cerrilla and Martínez, 2003). *In vitro* methods that utilize rumen fluid as part of the incubation procedure, however, could also be susceptible to between-study variation like the *in situ* technique because enzymatic activity in rumen fluid has been shown to be strongly influenced by animal diet (Giesecke and Geiges, 1974; Cone et al., 1989). A potential advantage of a dependable enzymatic *in vitro* method over the traditional *in situ* technique and rumen fluid culture-based *in vitro* technique may be that estimates of starch disappearance are not biased by factors such as particle loss or inconsistent enzymatic activity.

Conclusions

In this study, we presented an *in vitro* technique for determining starch degradation rates

of a wide selection of feeds and demonstrated a relationship to values obtained *in situ* when feed nutrient components were accounted for. The relationship between the *in vitro* and *in situ* measurements suggests the *in vitro* approach has potential use as laboratory technique to understand relative differences in starch degradability associated with different feedstuffs. The significant effects of various feed components on starch degradation kinetics indicate a necessity to account for nutrient content when predicting starch degradability using non-animal methods, and the large difference in range of estimates suggests the *in vitro* values are not directly biologically interpretable. Future work should focus on determining the repeatability of this assay and potential relationships to true *in vivo* estimations of starch degradation.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Mary Beth Hall for her intellectual contributions to this work. This research was supported by funding from the U.S. Department of Agriculture–National Institute of Food and Agriculture (2017-05943), Cooperative Research Farms, and the Pratt Endowment of Virginia Tech.

LITERATURE CITED

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67:1–48. doi:10.18637/jss.v067.i01.

Boerman, J.P., S.B. Potts, M.J. VandeHaar, M.S. Allen, and A.L. Lock. 2015. Milk production responses to a change in dietary starch concentration vary by production level in dairy cattle. J. Dairy Sci. 98:4698–4706. doi:10.3168/jds.2014-8999.

Bruno-Soares, A.M., J.M.F. Abreu, C.V.M. Guedes, and A.A. Dias-da-Silva. 2000. Chemical composition, DM and NDF degradation kinetics in rumen of seven legume straws. Anim. Feed Sci. Technol. 83:75–80. doi:10.1016/S0377-8401(99)00113-3.

Cerrilla, M.E.O., and G.M. Martínez. 2003. Starch digestion and glucose metabolism in the ruminant: a review. Interciencia 28:380–386.

Cone, J.W., W. Cliné-Theil, A. Malestein, and A.T. van ’t Klooster. 1989. Degradation of starch by incubation with rumen fluid. A comparison of different starch sources. J. Sci. Food Agric. 49:173–183. doi:10.1002/jsfa.2740490206.

Ewing, D.L., and D.E. Johnson. 1987. Corn particle starch digestion, passage and size reduction in beef steers: a dynamic model. J. Anim. Sci. 64:1194–1204. doi:10.2527/jas1987.6441194x.

French, D. 1973. Chemical and physical properties of starch. J. Anim. Sci. 37:1048–1061. doi:10.2527/jas1973.3741048x.

Fukushima, R.S., M.S. Kerley, M.H. Ramos, J.H. Porter, and R.L. Kallenbach. 2015. Comparison of acetyl bromide lignin with acid detergent lignin and Klason lignin and correlation with in vitro forage degradability. Anim. Feed Sci. Technol. 201:25–37. doi:10.1016/j.anifeedsci.2014.12.007.

Giesecke, D., and R. Geiges. 1974. Untersuchungen zur Genese und Biochemie der Pansenacidose 1. Stärke, Amylase-Aktivität und Acidität. Zentralblatt Für Veterinärmedizin Reihe A 21:261–267. doi:10.1111/j.1439-0442.1974.tb01119.x.

Grant, R. 2005. Optimizing starch concentrations in dairy rations. Pages 73–79 in Proceedings of the Tri-State Dairy Nutrition Conference, Fort Wayne, IN.

Hall, M.B. 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method: Collaborative study. J. AOAC Int. 98:397–409. doi:10.5740/jaoacint.15-012.

Hall, M.B., and G.B. Huntington. 2008. Nutrient synchrony: Sound in theory, elusive in practice. J. Anim. Sci. 86:E287–E292. doi:10.2527/jas.2007-0516.

Harmon, D.L. 2009. Understanding starch utilization in the small intestine of cattle. Asian-Australas. J. Anim. Sci. 22:915–922. doi:10.5713/ajas.2009.r.08.

Herrera-Saldana, R.E., J.T. Huber, and M.H. Poore. 1990. Dry matter, crude protein, and starch degradability of five cereal grains. J. Dairy Sci. 73:2386–2393. doi:10.3168/jds.S0022-0302(90)78922-9.

Hersom, M.J. 2008. Opportunities to enhance performance and efficiency through nutrient synchrony in forage-fed ruminants1. J. Anim. Sci. 86:E306–E317. doi:10.2527/jas.2007-0463.

Huhtanen, P., and J. Sveinbjörnsson. 2006. Evaluation of methods for estimating starch digestibility and digestion kinetics in ruminants. Anim. Feed Sci. Technol. 130:95–113. doi:10.1016/j.anifeedsci.2006.01.021.

Janicki, F.J., and C.C. Stallings. 1988. Degradation of crude protein in forages determined by in vitro and in situ procedures. J. Dairy Sci. 71:2440–2448. doi:10.3168/jds.S0022-0302(88)79829-X.

Kotarski, S.F., R.D. Waniska, and K.K. Thurn. 1992. Starch hydrolysis by the ruminal microflora. J. Nutr. 122:178–190. doi:10.1093/jn/122.1.178.

Laconi, E.B., and A. Jayanegara. 2015. Improving nutritional quality of cocoa pod (Theobroma cacao) through chemical and biological treatments for ruminant feeding: in vitro and in vivo evaluation. Asian-Australas. J. Anim. Sci. 28:343. doi:10.5713/ajas.13.0798.

Larsson, K., and Y. Miezis. 1979. On the possibility of dietary fiber formation by interaction in the intestine between starch and lipids. Starch-Stärke 31:301–302. doi:10.1002/star.19790310905.

McAllister, T.A., R.C. Phillippe, L.M. Rode, and K.-J. Cheng. 1993. Effect of the protein matrix on the digestion of cereal grains by ruminal microorganisms. J. Anim. Sci. 71:205–212. doi:10.2527/1993.711205x.

Michalet-Doreau, B., C. Philippeau, and M. Doreau. 1997. In situ and in vitro ruminal starch degradation of untreated and formaldehyde-treated wheat and maize. Reprod Nutr Dev 37:305–312. doi:10.1051/rnd:19970306.

Nocek, J.E. 1988. In situ and other methods to estimate ruminal protein and energy digestibility: a review. J. Dairy Sci. 71:2051–2069. doi:10.3168/jds.S0022-0302(88)79781-7.

Nocek, J.E., and J.E. English. 1986. In situ degradation kinetics: evaluation of rate determination procedure. J. Dairy Sci. 69:77–87. doi:10.3168/jds.S0022-0302(86)80372-1.

Nozière, P., and B. Michalet-Doreau. 1996. Validation of in sacco method: influence of sampling site, nylon bag or rumen contents, on fibrolytic activity of solid-associated microorganisms. Anim. Feed Sci. Technol. 57:203–210. doi:10.1016/0377-8401(95)00851-9.

Offner, A., A. Bach, and D. Sauvant. 2003. Quantitative review of in situ starch degradation in the rumen. Anim. Feed Sci. Technol. 106:81–93. doi:10.1016/S0377-8401(03)00038-5.

Ørskov, E.R., and I. McDonald. 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. J. Agric. Sci. 92:499–503. doi:10.1017/s0021859600063048.

Panyoo, A.E., and M.N. Emmambux. 2017. Amylose–lipid complex production and potential health benefits: A mini-review. Starch-Stärke 69:1600203. doi:10.1002/star.201600203.

R Core Team. 2019. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Raab, L., B. Cafantaris, T. Jilg, and K.H. Menke. 1983. Rumen protein degradation and biosynthesis: 1. A new method for determination of protein degradation in rumen fluid in vitro. Br. J. Nutr. 50:569–582. doi:10.1079/bjn19830128.

Seifried, N., H. Steingaß, and M. Rodehutscord, 2015. In vitro and in situ evaluation of secondary starch particle losses from nylon bags during the incubation of different cereal grains. Anim. Feed Sci. Technol. 210:26–36. doi:10.1016/j.anifeedsci.2015.09.011

Sveinbjörnsson, J., M. Murphy, and P. Udén. 2007. In vitro evaluation of starch degradation from feeds with or without various heat treatments. Anim. Feed Sci. Technol. 132:171–185. doi:10.1016/j.anifeedsci.2006.03.018.

Theurer, C.B. 1986. Grain processing effects on starch utilization by ruminants. J. Anim. Sci. 63:1649–1662. doi:10.2527/jas1986.6351649x.

Varel, V.H., K.K. Kreikemeier, H.-J.G. Jung, and R.D. Hatfield. 1993. In vitro stimulation of forage fiber degradation by ruminal microorganisms with Aspergillus oryzae fermentation extract. Appl. Environ. Microbiol. 59:3171–3176. doi:10.1128/AEM.59.10.3171-3176.1993.

Wang, M., J. Jiang, Z.L. Tan, S.X. Tang, Z.H. Sun, and X.F. Han. 2009. In situ ruminal crude protein and starch degradation of three classes of feedstuffs in goats. J. Appl. Anim. Res. 36:23–28. doi:10.1080/09712119.2009.9707023.

Whiting, F. 1957. The effect of concentrate to hay ratio and other ration factors on the feedlot performance of beef cattle. Can. J. Anim. Sci. 37:50–57. doi:10.4141/cjas57-007.

Zebeli, Q., D. Mansmann, H. Steingass, and B.N. Ametaj. 2010. Balancing diets for physically effective fibre and ruminally degradable starch: A key to lower the risk of sub-acute rumen acidosis and improve productivity of dairy cattle. Livest. Sci. 127:1–10. doi:10.1016/j.livsci.2009.09.003.

TABLES

Table 1. Modified enzymatic incubation times[1]

Incubation number	Amylase incubation time, min	Amyloglucosidase incubation time, min
1	5	10
2	10	20
3	15	30
4	30	60
5	45	90
6	60	120

[1]The acetate buffer method of starch analysis (Hall, 2015) utilizes a 60 min amylase incubation and a 120 min amyloglucosidase incubation.

Table 2. Feed samples and their starch degradation rates determined *in vitro* and *in situ*

Sample number	Feed name[1]	Feed mill	Starch, g/kg	*In vitro* degradation rate, %/h	*In situ* degradation rate, %/h
1	DDGS #1	1	32.5	2.98	10.4
2	Soy hulls #1	1	11.7	25.6	5.02
3	Barley #1	1	637	203	2.04
4	Beet pulp	2	23.3	19.5	9.60
5	Soy hulls #2	2	20.2	84.1	2.57
6	Wheat middlings #1	2	285	222	26.2
7	DGGS #2	2	32.7	59.6	5.45
8	Canola meal #1	3	22.2	87.9	8.71
9	Canola meal #2	3	20.1	106	4.12
10	Peas #1	3	492	164	42.8
11	Peas #2	3	627	155	8.63
12	Oathulls	3	125	258	4.25
13	Barley #2	3	668	279	9.64
14	Alfalfa	3	52.1	53.6	3.73
15	Cornmeal #1	4	664	156	1.53
16	Cornmeal #2	4	559	168	11.3
17	Wheat middlings #2	4	341	174	22.5
18	Steam flaked corn #1	4	824	160	6.07
19	Steam flaked corn #2	4	793	117	3.84

[1]DDGS = dried distillers grains with solubles.

Table 3. Significant predictor variables for *in situ* starch degradation rate[1]

Variable	Parameter estimate	*P* value
Intercept	-1.02	0.0083
In vitro starch degradation rate	0.431	0.0092
Starch content	0.00839	0.020
Fat content	0.0336	0.016
NDF content	0.0104	0.033
Enzyme-free sample starch content	0.500	0.00083
In vitro starch degradation rate × starch content	-0.00505	0.012
In vitro starch degradation rate × NDF content	-0.00589	0.026
Fit statistics		
n	19	
CCC	0.85	
RMSE	0.051	

[1]NDF = neutral detergent fiber; CCC = concordance correlation coefficient; RMSE = root mean square error.

CHAPTER 4: Rumen Fermentation and Epithelial Gene Expression Responses to Diets Containing Ingredients Designed to Differ in Ruminally Degradable Protein and Fiber Supplies

This chapter will be published as: Gleason, C. B., L. M. Beckett, and R. R. White. Rumen fermentation and epithelial gene expression responses to diets containing ingredients designed to differ in ruminally degradable protein and fiber supplies. Journal of Animal Science.

ABSTRACT

Although a body of work exists relating ruminal volatile fatty acid (**VFA**) concentrations to diet composition and animal performance, comparatively minimal information is available describing how VFA synthesis, absorption, and interconversion respond to diets within the context of the whole rumen environment. The objective of this study was to characterize how protein and fiber sources affect dry matter intake, rumen pH, fluid dynamics, fermentation parameters, and epithelial gene expression. Four diet treatments (soybean meal or heat-treated soybean meal and beet pulp or timothy hay) were delivered to 10 ruminally cannulated wethers (Suffolk, Dorset, or Suffolk x Dorset) using a partially replicated 4x4 Latin square design. The soybean meals served to deliver varying rumen degradabilities of crude protein (**CP**) while the beet pulp and timothy hay represented different rumen degradabilities of neutral detergent fiber (**NDF**). Dry matter intake, rumen pH, fluid pool size, and fluid passage rate were unaffected by treatment ($P > 0.05$). Rumen VFA concentrations of acetate, propionate, butyrate, valerate, and isovalerate and molar proportions of isovalerate were greater on beet pulp compared to timothy hay ($P < 0.05$) but were unaffected by CP source ($P > 0.05$). Butyrate synthesis ($P = 0.015$) and absorption ($P = 0.045$) were greater on the beet pulp treatment whereas synthesis and absorption of the other VFA remained unchanged ($P > 0.05$). Both CP and NDF treatment effects were associated with numerous VFA interconversions, predominantly in the conversions from acetate, propionate, and butyrate, with limited impact on the conversions from valerate and isobutyrate. No effects were observed for conversions from isovalerate. Expression levels of rumen epithelial genes involved in ion transportation, VFA metabolism, and epithelial integrity were not altered by diet treatment ($P > 0.05$). However, AKT1 and NHE2 tended to increase ($P = 0.088$ and $P = 0.075$, respectively) with beet pulp treatment compared to timothy hay. These results indicate

that rumen VFA concentrations and dynamics are altered by changes in dietary sources of nutrients but that animal intake, rumen environmental parameters, and the rumen epithelium may be less responsive to such changes.

Keywords: nutrients, rumen epithelium, sheep, volatile fatty acids

INTRODUCTION

Volatile fatty acids (**VFA**) produced by microbial fermentation of feed substrates in the rumen represent a key form of energy for the ruminant host animal as they supply approximately 70% of the metabolic energy that may be used for productive functions, such as growth and milk production (Bergman, 1990). The VFA profile produced in the rumen may exert a considerable degree of influence on the animal's energy utilization for such activities because the VFA are utilized at differing efficiencies (Thomas and Martin, 1988). Improving our understanding of factors that determine fermentative outcomes would therefore help us to optimize the productive efficiency of ruminant livestock. Diet characteristics, such as forage-to-concentrate ratio are known to influence VFA profiles and other variables that in turn may impact the VFA present in the rumen, including feed intake, rumen fluid dynamics, rumen pH, and rumen epithelial characteristics (Dijkstra et al., 2005; Yan et al., 2014). Studies examining diet effects on VFA have generally reported and discussed VFA responses to diet in terms of VFA concentrations. Concentrations, however, may be altered by fluid fluxes and should not be used for assessing or making predictions regarding dietary effects on fermentation parameters (Hall et al., 2015). Research efforts should instead aim to characterize VFA responses to diet in terms of their production, interconversion, and absorption. These dynamic fluxes may be modeled using data obtained through stable isotope dilution experiments. To date, few published investigations have examined the effects of differing nutrient sources on VFA dynamics, rumen fluid dynamics, rumen pH, and expression of key epithelial genes concurrently. This lack of a holistic understanding of fermentation parameters represents a primary gap in our knowledge of rumen function. There is also currently a deficiency of data on fermentation dynamics of minor VFA. Although only present in the rumen in small quantities, the branched-chain minor VFA

isobutyrate and isovalerate serve as essential substrate for certain rumen microbial species, and are used in the synthesis of other branched-chain compounds such as amino acids and fatty acids (Allison, 1969; Cotta and Hespell, 1986). The objective of this study was therefore to characterize how differing dietary sources of protein and fiber affect dry matter intake, rumen pH, fluid dynamics, fermentation dynamics of major and minor VFA, and epithelial gene expression using an ovine model.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

All animal use and procedures in this study were approved by the Virginia Tech Institutional Animal Care and Use Committee (Protocol #18-096). Ten ruminally cannulated commercial wethers (Suffolk, Dorset or Suffolk x Dorset) were housed individually in covered pens at the Smithfield Farm, Virginia Tech, Blacksburg, VA. Wethers were approximately 1.5 years of age and an average of 62.1 ± 6.6 kg body weight (**BW**) at the start of the experiment. Wethers were blocked by initial BW and randomly assigned to treatments in a 4 x 4 Latin square with 2 wethers serving as alternates. Treatment assignments were arranged factorially (2 x 2) and included feedstuffs thought to supply differing rumen degradabilities of CP and NDF. Soybean meal (**SBM**) and heat-treated soybean meal (**HSBM**) represented the CP sources with high and low rumen degradabilities, respectively (NRC, 2016). These meals were pelleted along with alfalfa, corn, barley, wheat middlings, trace mineral salt, and a sheep vitamin-mineral premix. Pelleted beet pulp (**BP**) and long timothy hay (**TH**) were utilized as the NDF sources, with BP expected to undergo swifter fiber degradation compared to TH. Diets (Table 1) were prepared daily by combining the appropriate protein pellet with the appropriate fiber source to yield the 4

treatments: highly degradable CP plus lowly degradable NDF (**SBM-TH**), highly degradable CP plus highly degradable NDF **(SBM-BP**), lowly degradable CP plus lowly degradable NDF (**HSBM-TH**), and lowly degradable CP plus highly degradable NDF (**HSBM-BP**). Each animal consumed each of the 4 diets for a 21-d period. Animals were gradually adapted to their diets during the first 3 d then consumed 100% of the diet for the remaining 18 d. Diets were provided once daily at 0800 h from d 0-15. Animals were fed 1/12th of their daily ration every 2 h for the remainder of the period in an effort to more closely mimic a metabolic steady state. Refusals were collected daily throughout the experiment to calculate dry matter intake (**DMI**). Clean, fresh water was available at all times.

Feed Analysis

Feed samples were dried for 24 h at 55°C in a forced-air oven (Thermo Scientific Heratherm Advanced Protocol Ovens Model 51028115, Fisher Scientific, Waltham, MA) and ground to pass through a 1 mm screen of a Wiley mill (Model 4, Thomas Scientific, Swedesboro, NJ). Dry matter percentage was determined by drying ground samples for 12 h at 100°C. Ash content was obtained through combustion in a muffle furnace (Sybron Thermolyne FA1730, Fisher Scientific, Waltham, MA) for 12 h at 500°C. Concentrations of NDF and acid detergent fiber were determined using the Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY). Sodium sulfite (Sodium Sulfite, anhydrous, Fisher Scientific, Waltham, MA) and α-amylase from *Bacillus licheniformis* (Thermostable Amylase HTL, BIO-CAT, Troy, VA) were utilized in the NDF analysis (Van Soest et al., 1991). Residues from ADF analysis were agitated for 3 h in 72% sulfuric acid in a 2 L beaker on a rocking platform (Flask Dancer, Boekel Scientific, Feasterville-Trevose, PA) to obtain acid detergent lignin concentrations. Nitrogen content was determined by combustion analysis using a Vario El Cube CN analyzer (Elementar

Americas Inc., Mount Laurel, NJ) and CP concentration was calculated as N percentage × 6.25. Starch concentration was determined following the acetate buffer method (Hall, 2015) with α-amylase and amyloglucosidase (E-AMGDF, Megazyme International, Wicklow, Ireland).

Ruminal Infusions and Fluid Sampling

On day 16 of each experimental period, each animal was fitted with 2 rumen fluid sampling devices consisting of tygon tubing terminating in a plastic mesh pot scrubber weighted with steel nuts. One pot scrubber was placed in the cranial portion of the rumen and the other in the caudal portion with the free ends of tygon tubing exiting the rumen via holes drilled in the cannula plug. Rumen fluid was collected by withdrawing 2.5 mL from both tube ends using a 60 mL luer lok syringe. The sample would then be aliquoted into 2 glass vials and frozen at -21°C until analysis. A 113 mL polyethylene glycol (**PEG**) (average MW 8000; FisherScientific, Hampton, NH) bolus (11.2 g PEG dissolved in 100 mL of water) was administered intraruminally via the rumen cannula at 0500 h on d 17 to 19. Continuous intraruminal infusions of VFA isotope were carried out using Plum A+ infusion pumps (Abbott Laboratories, IL, USA). Isotope solutions were infused individually at a rate of 100 mL/h for 6 h with randomization of infusion order across animals. Morning infusions began immediately after PEG bolusing at 0500 h and afternoon infusions began at 1400 h. The following isotopes (Cambridge Isotope Laboratories, Andover, MA) were utilized: Na-2-^{13}C-acetate (99% enrichment; solution concentration: 0.052%), Na-2-^{13}C-propionate (99% enrichment; solution concentration: 0.052%), Na-2-^{13}C-butyrate (99% enrichment; solution concentration: 0.026%), 2-^{13}C-valeric acid (99% enrichment; solution concentration: 0.013%), 2-^{13}C-isovaleric acid (99% enrichment; solution concentration: 0.0052%), and 2-^{13}C-isobutyric acid (99% enrichment; solution concentration: 0.0052%). The isotope infusions were delivered via an IV line attached to a third piece of tygon

tubing which was attached to a polyethylene bottle suspended in the rumen. The bottle was drilled with numerous holes in the sides to allow diffusion of the isotope solution and was weighted to maintain a central, upright position in the rumen. Rumen fluid samples were collected at 0430 h, 0445 h, and immediately after the PEG bolus at 0500 h, then hourly until 2300 h on d 17 to 19.

Determining Rumen Fluid Volume, Passage Rate, and pH

Polyethylene glycol concentrations of samples taken at 0500 through 1900 h on d 17 of each period were determined following a protocol modification of Smith (1959). Concentrations of PEG were then fitted to an exponential decay curve, the slope of which was taken as the fractional fluid passage rate. An estimation of rumen fluid volume was calculated by dividing the PEG bolus dose by the curve's y-intercept. Rumen fluid pH was measured on samples taken at 1100 h and 1700 h on d 17-19 using a handheld pH meter (Oakton pH 50 Spear Waterproof Pocket pH Testr, Fisher Scientific, Hampton, NH) immediately following collection.

Measurement of VFA Concentrations

A composite rumen fluid sample was created for each animal using 100 μL of each sample collected during a period day for a total of approximately 2 mL. Volatile fatty acids in each composite sample were derivatized and the concentrations determined by gas chromatography following a procedure adapted from Kristensen (2000). Derivatized samples were analyzed using a Thermo Electron Polaris Q mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) in tandem with a Thermo Electron Focus gas chromatograph (Thermo Fisher Scientific Inc., Waltham, MA) with XCalibur software version 1.4 (Thermo Fisher Scientific Inc., Waltham, MA). A Varian FactorFour capillary column VF-170ms (30 m, 0.25

mm, 0.25 μm) was utilized for VFA differentiation. One microliter of derivatized sample was loaded with the inlet temperature at 225°C on a split ratio of 80 running a constant flow of helium carrier gas set to 1.2 mL/min. The gas chromatograph was initiated at a temperature of 75°C, ramped at 5°C/min to 135°C, then at 40°C/min to 225°C. The mass spectrometer was programmed to run in positive selected ion monitoring mode, collecting 3 consecutive segment *m*/*z* pairs for acetate (43, 47), propionate (57, 59), and butyrate (71, 73) in that elution order. The processing method used to integrate the area under the curves for each *m*/*z* used the algorithm of the International Conference on Computer and Information Science.

Isotope Ratio Determination and Model Fitting

Four animals (each receiving the 4 diets in a unique sequence) were selected for isotope ratio determination of their rumen fluid samples. Isotope ratios were determined on individual unpooled samples after acidification with HCl. ^{13}C enrichment of the CO_2 produced from each VFA was assessed using an isotope ratio mass spectrometer (IRMS; Delta V, Thermo Fisher Scientific, Waltham, MA) coupled to a gas chromatograph via a combustion oven (GC-comb-IRMS). Volatile fatty acids were introduced into the GC using a SPME method (SPME autosampler kit for Thermo Tri-Plus; SPME Fiber Assembly, 75um CAR/PDMS, 23ga, Autosampler (Supelco, P/N 57343-U); SPME Liner for TQ, 0.8 mm ID, Straight Through (Supleco, PN 2876601-U), Thermo Scientific). The SPME fiber was exposed to the sample vial's headspace after heating of the sample at 240°C for 1 min. The VFA were separated on a Zebron ZB-FFAP column, 30 m × 0.25 mm × 0.25 um (Phenomenex, P/N 7HG-G009-11) operated at 300°C using helium as a carrier gas at a flow rate of 1.5 mL/min and the data yielded by the analysis were expressed as isotope ratios. Estimates of VFA production, interconversion, and absorption rates were derived by using the isotope ratios, VFA concentrations, and rumen fluid

volume and passage rate data to fit a dynamic mechanistic model of these VFA fluxes (Bedford et al., 2020). The standard 3-pool interchanging model (Nolan et al., 2014) was updated to reflect a 6-pool system with minor VFA. Volatile fatty acid production and interconversions were estimated by model fitting and passage of VFA from the rumen via fluid was determined using VFA concentration data and fluid passage measurements. Ruminal absorption of VFA was calculated as the VFA produced or interconverted but not passed with fluid. The FME package of R (R Core Team, 2019) was used to fit individual models for each infusion of each of the 4 selected animals.

Papillae Collection and Gene Expression Analysis

Rumen papillae biopsies were collected on d 20 by first inverting a small portion of the ventral sac of the rumen through the cannula and severing 60 to 80 papillae from the epithelium with cuticle scissors. Phosphate buffered saline (Potassium Phosphate Monobasic 210 mg/L; Sodium Chloride 9000 mg/L; Sodium Phosphate Dibasic, 726 mg/L, Gibco Life Technologies, Grand Island, NY) was used to clean rumen fluid and debris from the papillae before collection. Severed papillae were immediately placed into 2 cryovials containing RNALater (QIAGEN, Valencia, CA) to avoid RNA degradation and were stored at -80°C. Isolation of RNA was performed using an RNA Plus Mini Kit (QIAGEN, Valencia, CA) and adequate quality of the RNA was confirmed by concentration (ng/μL) and 260/280 ratio. Complementary DNA (**cDNA**) was prepared from the RNA using an Applied Biosystem High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA) and synthesized on a Mastercycler. The genes selected for analysis by Real-Time reverse transcription quantitative PCR (**qRTPCR**) included those with significant roles in VFA transport, ion transport, VFA metabolism, or epithelial integrity. These 12 genes (Supplementary Table S1) were the monocarboxylate

transporters 1, 2, and 4 (**MCT1**, **MCT2**, **MCT4**), sodium-hydrogen exchanger isoforms 1, 2, and 3 (**NHE1**, **NHE2**, **NHE3**), beta-hydroxybutyrate dehydrogenase type 1 (**BDH1**), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (**HMGCS2**), heat shock protein 70 (**HSP70**), serine-threonine protein kinase (**AKT1**), Claudin-1 (**CLDN1**), and gap-junction protein alpha 1 (**GJA1**). Ribosomal protein subunit 9 (**RPS9**) was utilized as the housekeeper gene to be run with all subsequent genes for comparison. All Real-Time qRTPCR was performed following the method of Lu et al. (2017). Briefly, 1.0 μL of cDNA combined with 9.0 μL of a primer Master Mix was pipetted onto an ABI MicroAmp Fast Optical 96-well reaction plate (Fisher 43-469-07, Fisher Scientific, Hampton, NH) in triplicate and covered with an optical adhesive cover. The Master Mix was created by combining 0.5 μL of the target gene's forward primer, 0.5 μL of the target gene's reverse primer, 5.0 μL of SYBR Fast Green Master Mix (Fisher 43-856-12, Fisher Scientific, Hampton, NH), and 3.0 μL of molecular-grade water. Plates were run on the Real-Time Fast machine (Applied Biosystems 7500 Real-Time PCR, Thermo Fisher Scientific, Waltham, MA).

Statistical Analysis

Statistical analyses were conducted in R version 3.6.1 (R Core Team, 2019) using the nlme package (Pinheiro et al., 2020). Response variables included DMI (kg/d); fluid volume (L); fluid passage rate (%/h); fluid pH; VFA concentrations (mM); VFA molar proportions (% mol); VFA synthesis, interconversion, and absorption rates (mmol/mmol/h); VFA fluxes (mmol/h); and epithelial gene expression ($2^{-\Delta Ct}$). Response variables were analyzed using the following linear model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + c_k + d_l + e_{ijkl},$$

where μ represents the overall mean, α_i is the effect of the i^{th} protein source, β_j is the effect of the j^{th} fiber source, $\alpha\beta_{ij}$ is the interaction of protein source i and fiber source j, c_k is the random effect of animal k, d_l is the random effect of period l, and e_{ijkl} is the residual error associated with protein source i, fiber source j, animal k, and period l. A number of residual error variance structures, including 1st Order Autoregressive, Unstructured, and Compound Symmetry, were compared for each of the response variables. Model quality was determined based on Akaike information criterion (**AIC**) and the model possessing the lowest AIC was selected for significance testing. Analysis of variance (**ANOVA**) was performed on each model and least square means calculated. Significance level was set at $P < 0.05$ and a tendency considered when $0.05 \leq P < 0.10$.

RESULTS AND DISCUSSION

Dry Matter Intake, Rumen pH, and Rumen Fluid Parameters

Mean DMI for each treatment group are given in Table 2. Dry matter intakes were unaffected by protein source ($P = 0.59$) and fiber source ($P = 0.96$), suggesting the diets had similar palatabilities despite differences in particle size across fiber sources caused by the inclusion of either pelleted beet pulp or long grass hay. Diets were formulated to contain the same percentages of CP, NDF, and starch so the lack of difference in intakes may also indicate that the fermentation products of the diets elicited similar satiety feedback responses in the brain. Herrera-Saldana and Huber (1989) also reported no effect on intake when ingredients representing differing degradabilities of protein were fed. Beckett et al. (2021), however, reported decreased DMI in Holstein heifers on beet pulp compared to timothy hay and identified palatability as a likely cause of the intake difference. The experiment of Beckett et al. (2021)

utilized a different form of timothy hay than we did (pelleted vs. long hay) and a different species, which may have contributed to the inconsistencies among observations.

Similarly to DMI, rumen pH was also unaffected by protein source or fiber source (P = 0.46 and P = 0.13, respectively; Table 2). A non-significant increase in rumen pH was observed on TH diets compared to BP diets (5.89 and 5.91 vs. 5.72 and 5.81; Table 2). This was not surprising as the physically effective NDF from forages and the lower digestibility of such NDF sources are known to help maintain a higher rumen pH by stimulating the production of bicarbonate-containing saliva during chewing, and by avoiding shifts towards more extreme rates of VFA production, which can occur when soluble carbohydrates are provided in abundance (Fox and Tedeschi, 2002; Beauchemin and Yang, 2005; White et al., 2017). The neutral detergent soluble fiber (**NDSF**) fraction of beet pulp is predominantly pectin and, as a feedstuff, beet pulp contributes virtually no physically effective fiber when included in the diet (Münnich et al., 2017). Despite this, however, beet pulp has demonstrated the ability to stabilize rumen pH (Mojtahedi and Danesh Mesgaran, 2011). Pectin fermentation does not produce lactate and generally occurs to a lesser extent as pH decreases (Strobel and Russell, 1986; Münnich et al., 2017). This is likely why we did not observe a significant difference in rumen pH values. Sheep are generally fed forage-heavy diets with little added concentrate, and normal rumen pH in sheep ranges from 6.4 to 6.8 (Jasmin et al., 2011). The diet treatments in this study were balanced to include 25% starch and, although mean rumen pHs were lower than the normal range, they were not low enough for the animals to be considered in a state of subacute ruminal acidosis (Kleen et al., 2003; Jasmin et al., 2011).

Rumen fluid volume and passage rate also demonstrated no response to protein source (P = 0.37 and P = 0.60, respectively; Table 2) or fiber source (P = 0.45 and P = 0.16, respectively;

Table 2). Rumen fluid volume and passage rate can be influenced by factors such as osmolality (from fluid flux across the rumen wall) and water intake (Argyle and Baldwin, 1988). Although these variables were not measured in our study, it appears that our diet treatments did not exert a noticeable influence on them. Links between water intake and DMI have been reported previously (Winchester and Morris, 1956). Differences in water intake were not expected and we did not observe differences in DMI among our treatments, which may indirectly help to explain why there were no differences in rumen fluid volumes or passage rates.

Fermentation Parameters

Concentrations of all VFA except isobutyrate were observed to increase in response to BP treatment compared to TH ($P < 0.006$; Table 3). No concentrations of VFA demonstrated a response to protein source ($P > 0.10$; Table 3). Although VFA concentrations may be used to describe effects of diet treatments under specific ruminal conditions, their sensitivity to fluid inflow and outflow make them inappropriate measurements for drawing general conclusions about dietary influences of fermentation or for attempting to make comparisons across different studies (Hall et al., 2015). Concentrations of VFA were calculated for this investigation as an input for the VFA dynamics modeling process and their values are presented in this writing only for consistency with reporting practices in the literature. The remainder of this discussion will therefore focus on describing dietary impacts on ruminal VFA in terms of their molar proportions and fermentation dynamics rather than concentrations.

Volatile fatty acid molar proportions were minimally impacted by treatment. The only significant effect was observed for isovalerate molar proportions, which were greater on the BP treatment compared to TH ($P = 0.0065$; Table 3). The BP treatment also tended to increase molar

proportions of valerate ($P = 0.056$) whereas TH was associated with trends for greater percentages of acetate and isobutyrate ($P = 0.090$ and $P = 0.079$, respectively). These results are mostly consistent with the observations of Beckett et al. (2021), who reported greater acetate proportion in response to TH, a tendency for isobutyrate proportion to increase on TH, and a tendency for valerate proportion to increase on BP. Interestingly, Beckett and colleagues (2021) observed isovalerate proportions to increase on TH instead of BP, in direct conflict with our observation. Even though isovalerate only represents a minor proportion of the rumen VFA, it is an important substrate used by rumen microbes to synthesize leucine and other branched-chain compounds (Allison, 1969). The inconsistency of isovalerate response to these differing fiber treatments therefore merits further investigation. The overall lack of significance for the other VFA proportions, however, indicates that the rumen microbes are able to use different substrate sources to generate fairly similar fermentative profiles.

Butyrate synthesis rate and absorption fluxes increased in response to treatment with BP compared to TH ($P = 0.015$ and $P = 0.045$, respectively; Table 4). Synthesis and absorption of other VFA were not affected by diet treatment, nor was diet associated with altered fluid-mediated exit of any VFA (Table 4). This suggests that changes in dietary sources of fiber and protein may not alter rumen function sufficiently to elicit shifts in the production, absorption, or outflow of most VFA. Investigations examining dietary effects on VFA production and absorption rates are scarce, making extensive comparisons with our findings to the current available literature difficult. Esdale and colleagues (1968) reported production rate increases in both butyrate and propionate when corn silage was fed compared to alfalfa hay. Sutton et al. (2003) similarly observed considerable increases in the rate of propionate production as diet concentrate level was increased but, interestingly, reported a slight decrease in the rate of

butyrate production. While the diet treatments utilized in these two investigations would be expected to contain differing concentrations of nutrients such as starch and protein (unlike our diets), they nevertheless indicate that rates of butyrate production are sensitive to diet characteristics. In addition to pectin, the carbohydrate fraction of beet pulp is composed largely of hemicellulose (Münnich et al., 2018), which is a preferred fermentative substrate of the rumen bacterial species *Butyrivibrio fibrisolvens* (Emerson and Weimer, 2017). As its name suggests, *B. fibrisolvens* is a prominent producer of butyrate (Emerson and Weimer, 2017). It is therefore possible that the BP diet treatment stimulated an increase in butyrate synthesis through the provision of hemicellulose to a key butyrate-producing bacterial population. Of the ruminal VFA fermented, butyrate is the one primarily metabolized by the rumen epithelium as an energy source (Rémond et al., 1995). The increased absorption of butyrate observed in response to the BP treatment suggests that the epithelium may be altered to increase its uptake of this VFA; however, as will be discussed below, no significant changes in rumen epithelial gene expression due to diet treatment (including any associated with VFA transport) were observed. Regardless of the exact microbial or epithelial mechanisms potentially in play, our results demonstrate that butyrate synthesis and absorption may be altered by differences in the dietary source of fiber provided to the animal.

Contrasting with the lack of dietary effect on many of our other response variables of interest, numerous changes in VFA interconversion rates and fluxes were observed in response to both fiber and protein treatments (Table 5). Treatment with BP resulted or tended to result in greater fluxes of carbon towards isovalerate from acetate ($P = 0.0064$), propionate ($P = 0.0016$), valerate ($P = 0.0014$), and isobutyrate ($P = 0.091$). The increased shift towards isovalerate helps to explain the greater molar proportions observed for this VFA on the BP treatment. Rates of

interconversion from acetate and propionate to isovalerate tended to increase as well ($P = 0.059$ and $P = 0.075$, respectively). Beet pulp treatment was also associated with an increased flux from acetate to valerate ($P = 0.0097$) and a tendency for increased flux from isobutyrate to propionate ($P = 0.075$). Interconversion rates observed to increase or tending to increase when TH diets were fed were predominantly involving propionate. These exchanges were the fractional interconversion rate from acetate to propionate ($P = 0.0031$), propionate to butyrate ($P = 0.088$), and propionate to valerate ($P = 0.0080$). The flux from acetate to propionate was also increased ($P = 0.015$). Interconversion rates that increased or tended to increase in response to the HSBM treatment compared to the SBM treatment included propionate to valerate ($P = 0.023$), valerate to isovalerate ($P = 0.096$), and isobutyrate to butyrate ($P = 0.047$). The corresponding fluxes from valerate to isovalerate and isobutyrate to butyrate were greater as well ($P = 0.030$ and $P = 0.035$, respectively). The SBM treatment tended to increase the rate of transfer from butyrate to acetate and propionate ($P = 0.061$ and $P = 0.055$, respectively).

Previous investigations have established that VFA interconversions are impacted to some extent by diet characteristics. Esdale et al. (1968) reported increased carbon transfer from acetate to butyrate associated with feeding corn silage compared to alfalfa hay. Minimal interconversion with propionate was observed on either treatment (Esdale et al., 1968). Work by Sutton et al. (2003), however, found that 13% and 10% of butyrate carbon originated from propionate at diet levels of 60% and 90% concentrate, respectively. An additional investigation utilizing high and low forage-to-concentrate ratios as diet treatments described fluxes from acetate to propionate and acetate to butyrate, with minimal conversion from butyrate back to acetate (Markantonatos et al., 2008). The explanatory power of these findings is limited by the fact that all of the diet treatments essentially only represent varying levels of forage-to-concentrate ratio. However, they

still illustrate that differences in fermentative substrate type can influence fluxes between the three major VFA. To the best of our knowledge, no published studies have reported diet effects on interconversions involving the minor rumen VFA. Our results therefore appear to be among the first characterizations to be made available of diet-driven alterations in the interconversion dynamics between both major and minor VFA.

The precise manner in which the differences in dietary sources of fiber and protein are altering the exchanges between the various VFA is not readily apparent, but it is probable that diet is influencing ruminal variables not examined in this study that play a role in driving interconversions. As explained by Ungerfeld and Kohn (2006) using thermodynamics principles, rumen VFA interconversions are able to occur because the change in Gibbs free energy (**ΔG**) for the VFA interconversion reactions can be inferred to be near zero and, thus, near equilibrium based on the fact that the reactions producing acetate, propionate, and butyrate from pyruvate have similar ΔG values. The ability of VFA to interconvert is known to be an important tool utilized by rumen microbes to regenerate intermediates needed for particular metabolic processes (Hackmann and Firkins, 2015). A possible explanation for the diet-induced responses in interconversions we observed is that the microbes are adapting to changes in the ruminal availability of fermentative substrates provided by our particular treatments by adjusting aspects of their VFA interconversion activities. Additional investigation is required to determine if this is indeed the route by which diet is exerting influence on interconversion dynamics.

Epithelial Gene Expression

Expression levels of the rumen epithelial genes under investigation were not significantly altered by treatment ($P > 0.05$; Table 6). However, expression of the genes AKT1 and NHE2

tended to increase (P = 0.088 and P = 0.075; respectively) in response to the BP treatment compared to TH. AKT1 codes for Serine-threonine protein kinase B, an enzyme involved in protein synthesis regulation (Wang and Proud, 2006). Specific dietary influences of AKT1 gene expression in the rumen epithelium are largely unknown. Recent investigations into a possible link between AKT1 and feed intake have yielded conflicting results (Bedford et al., 2020; Xue et al., 2020). NHE2 is expressed within epithelial cells and is involved with the regulation of intracellular sodium levels and pH (Graham et al., 2007). Gene expression of NHE2 was found to be upregulated in a caprine model when diet concentrate fraction was increased (Yan et al., 2014), indicating this gene may be regulated by diet characteristics. Interestingly, Beckett and colleagues (2021) observed that a small number of epithelial gene expression levels were affected or tended to be affected by beet pulp vs. timothy hay treatment but these did not include AKT1 or NHE2. We, conversely, did not observe any alteration in expression of the genes identified by Beckett et al. (2021) as sensitive to fiber source (NHE3 and HSP70) or as showing a trend toward sensitivity to fiber source (BDH1 and MCT4). Beckett and colleagues (2021) also reported a difference in rumen pH associated with the fiber treatment. As mentioned above, we observed no treatment effect on rumen pH. Other investigations into epithelial gene expression have noted that dietary alterations associated with changes in gene transcription may be exerting their effects via the creation of acidic conditions in the rumen (Hollmann et al., 2013; Yan et al., 2014; Zhang et al., 2019). Together, these observations and ours indicate that changes in the type of fermentative substrate (e.g. fiber vs. starch) that result in ruminal pH shifts are more likely than changes in the source of the same nutrient (e.g. forage fiber vs. non-forage fiber) to alter epithelial gene expression. Our finding of little to no dietary effect on gene expression is important because it suggests that varying nutrient sources do not necessarily result in altered

epithelial function, which indicates a certain level of flexibility may be possible with the usage of particular diet components. It must be remembered, however, that while we can use gene expression levels to indicate changes in the molecular response to a treatment, we cannot infer the level of functional protein from them. Additional research is therefore necessary to elucidate any impacts of nutrient source alterations on the actual abundances of key proteins involved in rumen epithelial function.

Conclusions

This work sought to characterize the responses of dry matter intake, rumen fluid parameters, rumen pH, VFA dynamics, and rumen epithelial gene expression levels to diets differing in sources of fiber and protein. Using an ovine model, differences in dietary sources of fiber and protein were found to elicit shifts in rumen VFA profiles and dynamics, especially in interconversions among VFA. Treatment differences, however, may have been insufficient to exert a significant degree of change on feed intake, rumen environmental characteristics, and gene expression levels in the rumen epithelium. The complex, interrelated process of converting feed to VFA is currently not well understood, and our investigation helps to bridge this knowledge gap by providing novel descriptions of carbon exchanges with minor VFA as impacted by diet characteristics. Future work should investigate the effects of these diet variables on postabsorptive metabolism of VFA and animal performance parameters.

LITERATURE CITED

Allison, M. J. 1969. Biosynthesis of amino acids by ruminal microorganisms. J. Anim. Sci. 29:797–807. doi:10.2527/jas1969.295797x.

Argyle, J. L., and R. L. Baldwin. 1988. Modeling of rumen water kinetics and effects of rumen pH changes. J. Dairy Sci. 71:1178–1188. doi:10.3168/jds.S0022-0302(88)79672-1.

Beauchemin, K. A., and W. Z. Yang. 2005. Effects of physically effective fiber on intake, chewing activity, and ruminal acidosis for dairy cows fed diets based on corn silage. J. Dairy Sci. 88:2117–2129. doi:10.3168/jds.S0022-0302(05)72888-5.

Beckett, L. M., C. B. Gleason, A. Bedford, D. M. Liebe, T. Yohe, M. B. Hall, K. M. Daniels, and R. R. White. 2021. Rumen volatile fatty acid molar proportions, rumen epithelial gene expression, and blood metabolite concentration responses to ruminally degradable starch and fiber supplies. J. Dairy Sci. In Press.

Bedford, A., L. Beckett, L. Harthan, C. Wang, N. Jiang, H. Schramm, L. L. Guan, K. M. Daniels, M. D. Hanigan, and R. R. White. 2020. Ruminal volatile fatty acid absorption is affected by elevated ambient temperature. Sci. Rep. 10:13092. doi:10.1038/s41598-020-69915-x.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70:567–590. doi:10.1152/physrev.1990.70.2.567.

Cotta, M. A., and R. B. Hespell. 1986. Protein and amino acid metabolism of rumen bacteria. In: Proceedings of 6th International Symposium on Ruminant Physiology. Prentice-Hall, Banff, Canada. p. 10–14.

Dijkstra, J., J. M. Forbes, and J. France. 2005. Quantitative aspects of ruminant digestion and metabolism. Cabi.

Emerson, E. L., and P. J. Weimer. 2017. Fermentation of model hemicelluloses by Prevotella strains and Butyrivibrio fibrisolvens in pure culture and in ruminal enrichment cultures. Appl. Microbiol. Biotechnol. 101:4269–4278. doi:10.1007/s00253-017-8150-7.

Esdale, W. J., G. A. Broderick, and L. D. Satter. 1968. Measurement of ruminal volatile fatty acid production from alfalfa aay or corn silage rations using a continuous infusion isotope dilution technique. J. Dairy Sci. 51:1823–1830. doi:10.3168/jds.S0022-0302(68)87285-6.

Fox, D. G., and L. O. Tedeschi. 2002. Application of physically effective fiber in diets for feedlot cattle. In: Proceedings of the Plains Nutrition Conference. San Antonio, TX. p. 67–81.

Graham, C., I. Gatherar, I. Haslam, M. Glanville, and N. L. Simmons. 2007. Expression and localization of monocarboxylate transporters and sodium/proton exchangers in bovine rumen epithelium. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 292:R997–R1007. doi:10.1152/ajpregu.00343.2006.

Hackmann, T. J., and J. L. Firkins. 2015. Maximizing efficiency of rumen microbial protein production. Front. Microbiol. 6:465. doi:10.3389/fmicb.2015.00465.

Hall, M. B., T. D. Nennich, P. H. Doane, and G. E. Brink. 2015. Total volatile fatty acid concentrations are unreliable estimators of treatment effects on ruminal fermentation in vivo. J. Dairy Sci. S0022030215002088. doi:10.3168/jds.2014-8854.

Herrera-Saldana, R., and J. T. Huber. 1989. Influence of varying protein and starch degradabilities on performance of lactating cows. J. Dairy Sci. 72:1477–1483. doi:10.3168/jds.S0022-0302(89)79257-2.

Hollmann, M., I. Miller, K. Hummel, S. Sabitzer, B. U. Metzler-Zebeli, E. Razzazi-Fazeli, and Q. Zebeli. 2013. Downregulation of cellular protective factors of rumen epithelium in goats fed high energy diet. J. Padmanabhan, editor. PLoS ONE. 8:e81602. doi:10.1371/journal.pone.0081602.

Jasmin, B. H., R. C. Boston, R. B. Modesto, and T. P. Schaer. 2011. Perioperative ruminal pH changes in domestic sheep (Ovis aries) housed in a biomedical research setting. J. Am. Assoc. Lab. Anim. Sci. 50:27–32.

Kleen, J. L., G. A. Hooijer, J. Rehage, and J. Noordhuizen. 2003. Subacute ruminal acidosis (SARA): A review. J. Vet. Med. Ser. A. 50:406–414.

Kristensen, N. B. 2000. Quantification of whole blood short-chain fatty acids by gas chromatographic determination of plasma 2-chloroethyl derivatives and correction for dilution space in erythrocytes. Acta Agric. Scand. Sect. - Anim. Sci. 50:231–236. doi:10.1080/090647000750069421.

Lu, Y., J. S. Bradley, S. R. McCoski, J. M. Gonzalez, A. D. Ealy, and S. E. Johnson. 2017. Reduced skeletal muscle fiber size following caloric restriction is associated with calpain-mediated proteolysis and attenuation of IGF-1 signaling. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 312:R806–R815. doi:10.1152/ajpregu.00400.2016.

Markantonatos, X., M. H. Green, and G. A. Varga. 2008. Use of compartmental analysis to study ruminal volatile fatty acid metabolism under steady state conditions in Holstein heifers. Anim. Feed Sci. Technol. 143:70–88. doi:10.1016/j.anifeedsci.2007.05.005.

Mojtahedi, M., and M. Danesh Mesgaran. 2011. Effects of the inclusion of dried molassed sugar beet pulp in a low-forage diet on the digestive process and blood biochemical parameters of Holstein steers. Livest. Sci. 141:95–103. doi:10.1016/j.livsci.2011.05.009.

Münnich, M., R. Khiaosa-ard, F. Klevenhusen, A. Hilpold, A. Khol-Parisini, and Q. Zebeli. 2017. A meta-analysis of feeding sugar beet pulp in dairy cows: Effects on feed intake, ruminal fermentation, performance, and net food production. Anim. Feed Sci. Technol. 224:78–89. doi:10.1016/j.anifeedsci.2016.12.015.

Münnich, M., A. Khol-Parisini, F. Klevenhusen, B. U. Metzler-Zebeli, and Q. Zebeli. 2018. Graded replacement of maize grain with molassed sugar beet pulp modulated ruminal microbial

community and fermentation profile in vitro: Sugar beet pulp, rumen microbiota and fermentation in vitro. J. Sci. Food Agric. 98:991–997. doi:10.1002/jsfa.8547.

Nolan, J. V., R. A. Leng, R. C. Dobos, and R. C. Boston. 2014. The production of acetate, propionate and butyrate in the rumen of sheep: Fitting models to 14C- or 13C-labelled tracer data to determine synthesis rates and interconversions. Anim. Prod. Sci. 54:2082. doi:10.1071/AN14539.

NRC. 2016. Nutrient requirements of beef cattle. 8th ed. National Academies Press, Washington, DC.

Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar, and R Core Team. 2020. nlme: Linear and nonlinear mixed effects models. R Foundation for Statistical Computing, Vienna, Austria. Available from: https://CRAN.R-project.org/package=nlme

R Core Team. 2019. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rémond, D., I. Ortigues, and J.-P. Jouany. 1995. Energy substrates for the rumen epithelium. Proc. Nutr. Soc. 54:95–105. doi:10.1079/PNS19950040.

Smith, R. H. 1959. The development and function of the rumen in milk-fed calves. J. Agric. Sci. 52:72–78. doi:10.1017/S0021859600035693.

Strobel, H. J., and J. B. Russell. 1986. Effect of pH and energy spilling on bacterial protein synthesis by carbohydrate-limited cultures of mixed rumen bacteria. J. Dairy Sci. 69:2941–2947. doi:10.3168/jds.S0022-0302(86)80750-0.

Sutton, J. D., M. S. Dhanoa, S. V. Morant, J. France, D. J. Napper, and E. Schuller. 2003. Rates of production of acetate, propionate, and butyrate in the rumen of lactating dairy cows given normal and low-roughage diets. J. Dairy Sci. 86:3620–3633. doi:10.3168/jds.S0022-0302(03)73968-X.

Thomas, P. C., and P. A. Martin. 1988. The influence of nutrient balance on milk yield and composition. In: Proceedings of the 46th University of Nottingham Easter School in Agricultural Sciences. Butterworths, London, UK. p. 97–118.

Ungerfeld, E. M., and R. A. Kohn. 2006. The role of thermodynamics in the control of ruminal fermentation. In: K. Sejrsen, T. Hvelplund, and M. O. Nielsen, editors. Ruminant physiology: Digestion, metabolism and impact of nutrition on gene expression, immunology and stress. Wageningen Academic, Wageningen, Netherlands. p. 55–85.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597. doi:10.3168/jds.s0022-0302(91)78551-2.

Wang, X., and C. G. Proud. 2006. The mTOR pathway in the control of protein synthesis. Physiology. 21:362–369. doi:10.1152/physiol.00024.2006.

White, R. R., M. B. Hall, J. L. Firkins, and P. J. Kononoff. 2017. Physically adjusted neutral detergent fiber system for lactating dairy cow rations. II: Development of feeding recommendations. J. Dairy Sci. 100:9569–9584. doi:10.3168/jds.2017-12766.

Winchester, C. F., and M. J. Morris. 1956. Water intake rates of cattle. J. Anim. Sci. 15:722–740. doi:10.2527/jas1956.153722x.

Xue, Y., L. Lin, F. Hu, W. Zhu, and S. Mao. 2020. Disruption of ruminal homeostasis by malnutrition involved in systemic ruminal microbiota-host interactions in a pregnant sheep model. Microbiome. 8:138. doi:10.1186/s40168-020-00916-8.

Yan, L., B. Zhang, and Z. Shen. 2014. Dietary modulation of the expression of genes involved in short-chain fatty acid absorption in the rumen epithelium is related to short-chain fatty acid concentration and pH in the rumen of goats. J. Dairy Sci. 97:5668–5675. doi:10.3168/jds.2013-7807.

Zhang, K., M. Meng, L. Gao, Y. Tu, and Y. Bai. 2019. Rumen-derived lipopolysaccharide induced ruminal epithelium barrier damage in goats fed a high-concentrate diet. Microb. Pathog. 131:81–86. doi:10.1016/j.micpath.2019.02.007.

TABLES

Table 1. Ingredients and nutrient inclusion for each treatment diet[1,2]

Ingredient, % of DM	SBM-TH	HSBM-TH	SBM-BP	HSBM-BP
Alfalfa hay	10.0	9.9	10.1	10.1
Corn	16.0	15.7	19.2	19.9
Barley	16.0	15.7	19.2	19.9
Soybean meal	13.9	0.00	16.1	0.00
Soyplus	0.00	14.4	0.00	18.1
Timothy hay	23.5	20.6	0.00	0.00
Beet pulp	0.00	0.00	42.0	41.0
Wheat middlings	20.0	23.0	2.20	0.50
Trace mineral salt	0.74	0.88	0.54	0.55
Vitamin premix	0.74	0.88	0.54	0.55
Nutrient, %[3]				
DM	96.2	96.2	96.2	96.2
CP	17.0	16.8	16.6	16.1
NDF	29.8	30.7	29.6	31.0
ADF	13.5	12.9	13.7	14.2
Lignin	2.11	1.80	1.96	2.19
Starch	27.3	26.6	27.9	24.9
Ash	4.87	4.22	4.79	4.96

[1]HSBM-TH = heat-treated soybean meal and timothy hay; SBM-TH = soybean meal and timothy hay; HSBM-BP = heat-treated soybean meal and beet pulp; SBM-BP = heat-treated soybean meal and beet pulp.
[2]Alfalfa hay, corn, barley, wheat middlings, trace mineral salt, and vitamin premix were incorporated into pellets along with either soybean meal or Soyplus to create the SBM and HSBM treatments, respectively.
[3]Nutrient percentages are expressed on a DM basis except for DM, which is expressed on an AF basis.

Table 2. LS means for DMI and rumen variables as differentiated by diet along with *P* values for the effects of protein source, fiber source, and the interaction of protein and fiber source[1]

	Diet					*P* value		
Item	SBM-TH	HSBM-TH	SBM-BP	HSBM-BP	SEM	Protein	Fiber	Protein × Fiber
DMI, kg	1.56	1.48	1.52	1.51	0.12	0.59	0.96	0.57
Rumen pH	5.89	5.91	5.72	5.81	0.079	0.46	0.13	0.66
Rumen fluid volume, L	2.34	3.37	3.35	3.50	0.75	0.37	0.45	0.56
Rumen fluid Passage rate, %/h	8.63	6.47	3.90	3.81	2.7	0.60	0.16	0.69

[1]HSBM-TH = heat-treated soybean meal and timothy hay; SBM-TH = soybean meal and timothy hay; HSBM-BP = heat-treated soybean meal and beet pulp; SBM-BP = heat-treated soybean meal and beet pulp.

Table 3. LS means for ruminal VFA concentrations and molar proportions as differentiated by diet along with *P* values for the effects of protein source, fiber source, and the interaction of protein and fiber source[1]

	Diet					*P* value		
Item	SBM-TH	HSBM-TH	SBM-BP	HSBM-BP	SEM	Protein	Fiber	Protein × Fiber
Concentration, mM								
Acetate	3.05	3.09	3.65	3.63	0.17	0.99	**<0.001**	0.84
Propionate	1.11	0.992	1.41	1.23	0.098	0.11	**0.0053**	0.76
Butyrate	0.582	0.668	0.846	0.897	0.076	0.37	**0.0036**	0.82
Valerate	0.0830	0.0808	0.126	0.121	0.012	0.76	**0.0019**	0.92
Isovalerate	0.0476	0.0499	0.0927	0.0740	0.0082	0.27	**<0.001**	0.17
Isobutyrate	0.0437	0.0461	0.0516	0.0438	0.0030	0.38	0.26	**0.081**
Molar proportion, % mol								
Acetate	63.1	62.7	60.5	61.6	1.3	0.77	**0.090**	0.47
Propionate	21.9	20.1	22.1	20.2	1.4	0.16	0.86	0.94
Butyrate	11.6	13.5	13.4	14.6	1.0	0.12	0.23	0.72
Valerate	1.65	1.65	1.96	1.94	0.15	0.97	**0.056**	0.91
Isovalerate	0.951	1.03	1.52	1.25	0.16	0.52	**0.0065**	0.24
Isobutyrate	0.873	0.964	0.852	0.781	0.067	0.80	**0.079**	0.16

[1]HSBM-TH = heat-treated soybean meal and timothy hay; SBM-TH = soybean meal and timothy hay; HSBM-BP = heat-treated soybean meal and beet pulp; SBM-BP = heat-treated soybean meal and beet pulp.

Table 4. LS means for VFA dynamics measurements as differentiated by diet along with *P* values for the effects of protein source, fiber source, and the interaction of protein and fiber source[1,2]

	Diet					*P* value		
Measurement	SBM-TH	HSBM-TH	SBM-BP	HSBM-BP	SEM	Protein	Fiber	Protein × Fiber
Synthesis, mmol/h								
Acetate	22.3	15.7	17.8	28.4	6.9	0.73	0.50	0.18
Propionate	12.3	4.44	6.11	14.2	6.0	0.99	0.77	0.20
Butyrate	1.53	1.07	2.87	7.41	1.6	0.15	**0.015**	**0.083**
Valerate	1.82×10^{-4}	1.09×10^{-5}	4.10×10^{-6}	6.33×10^{-5}	9.63×10^{-5}	0.57	0.53	0.26
Isovalerate	1.06×10^{-5}	9.52×10^{-5}	6.18×10^{-4}	7.96×10^{-4}	5.06×10^{-4}	0.80	0.22	0.93
Isobutyrate	4.65×10^{-5}	3.23×10^{-8}	4.10×10^{-9}	3.15×10^{-7}	2.33×10^{-5}	0.34	0.34	0.33
Absorption rate, mmol/mmol/h								
Acetate	0.755	0.591	0.716	0.699	0.090	0.34	0.70	0.69
Propionate	0.763	0.629	0.762	0.725	0.077	0.29	0.54	0.53
Butyrate	0.704	0.563	0.618	0.564	0.13	0.46	0.74	0.74
Valerate	0.741	0.578	0.745	0.644	0.11	0.26	0.76	0.78
Isovalerate	0.564	0.696	0.531	0.542	0.13	0.58	0.47	0.64
Isobutyrate	0.671	0.590	0.738	0.763	0.10	0.75	0.21	0.56
Absorbed, mmol/h								
Acetate	13.0	9.01	11.7	23.6	5.9	0.45	0.22	0.16
Propionate	9.33	4.27	6.47	12.6	4.1	0.90	0.52	0.20
Butyrate	3.11	3.65	4.27	7.45	1.3	0.12	**0.045**	0.25
Valerate	0.948	0.525	0.915	1.21	0.31	0.77	0.19	0.16
Isovalerate	0.229	0.506	0.564	0.643	0.20	0.34	0.22	0.59
Isobutyrate	0.387	0.324	0.429	0.785	0.21	0.39	0.16	0.23
Passage with fluid, mmol/h								
Acetate	4.31	2.24	1.14	1.53	2.2	0.69	0.36	0.56
Propionate	3.42	1.19	0.623	0.806	1.7	0.53	0.34	0.47
Butyrate	1.00	0.970	0.500	1.17	0.76	0.62	0.82	0.59
Valerate	0.338	0.180	0.0976	0.124	0.18	0.69	0.38	0.58
Isovalerate	0.112	0.103	0.0988	0.0800	0.083	0.84	0.80	0.94
Isobutyrate	0.117	0.0993	0.0401	0.0402	0.067	0.90	0.31	0.89

[1]HSBM-TH = heat-treated soybean meal and timothy hay; SBM-TH = soybean meal and timothy hay; HSBM-BP = heat-treated soybean meal and beet pulp; SBM-BP = heat-treated soybean meal and beet pulp.

[2]mmol/mmol/h = fraction of carbon per hour.

Table 5. LS means for significant and trending VFA interconversions as differentiated by diet along with *P* values for the effects of protein source, fiber source, and the interaction of protein and fiber source[1,2]

	Diet					*P* value		
Measurement	SBM-TH	HSBM-TH	SBM-BP	HSBM-BP	SEM	Protein	Fiber	Protein × Fiber
Interconversion rate, mmol/mmol/h								
Acetate to propionate	0.355	0.404	0.176	0.0972	0.063	0.82	**0.0031**	0.32
Acetate to isovalerate	0.0376	0.0245	0.0624	0.0593	0.014	0.57	**0.059**	0.73
Propionate to butyrate	0.222	0.478	0.224	0.151	0.11	0.31	**0.088**	**0.084**
Propionate to valerate	0.0582	0.0960	0.0323	0.0502	0.017	**0.023**	**0.0080**	0.32
Propionate to isovalerate	0.0133	0.0166	0.0341	0.0433	0.013	0.61	**0.075**	0.81
Butyrate to acetate	0.446	0.262	0.317	0.184	0.12	**0.061**	0.19	0.74
Butyrate to propionate	0.407	0.244	0.319	0.123	0.092	**0.055**	0.22	0.83
Valerate to acetate	0.477	0.261	0.203	0.451	0.12	0.90	0.73	**0.077**
Valerate to isovalerate	0.0286	0.0811	0.0494	0.132	0.037	**0.096**	0.34	0.68
Isobutyrate to butyrate	0.170	0.477	0.332	0.535	0.12	**0.047**	0.36	0.66
Interconversion flux, mmol/h								
Acetate to propionate	6.26	7.28	1.91	1.62	1.8	0.84	**0.015**	0.72
Acetate to valerate	1.17	0.521	1.15	1.75	0.36	0.89	**0.0097**	**0.0090**
Acetate to isovalerate	0.456	0.379	1.20	1.77	0.47	0.38	**0.0064**	0.26
Propionate to isovalerate	0.142	0.119	0.421	0.734	0.22	0.32	**0.016**	0.26
Valerate to isovalerate	0.0288	0.0614	0.0777	0.219	0.040	**0.030**	**0.014**	0.14
Isobutyrate to propionate	0.0740	0.0713	0.189	0.223	0.087	0.81	**0.075**	0.77
Isobutyrate to butyrate	0.0920	0.403	0.211	0.495	0.14	**0.035**	0.40	0.91
Isobutyrate to isovalerate	0.0163	0.0232	0.0515	0.0987	0.040	0.36	**0.091**	0.49

[1]HSBM-TH = heat-treated soybean meal and timothy hay; SBM-TH = soybean meal and timothy hay; HSBM-BP = heat-treated soybean meal and beet pulp; SBM-BP = heat-treated soybean meal and beet pulp.
[2]mmol/mmol/h = fraction of carbon per hour.

Table 6. LS means for rumen epithelial gene expression ($2^{-\Delta Ct}$) as differentiated by diet along with *P* values for the effects of protein source, fiber source, and the interaction of protein and fiber source[1]

Gene	Abbreviation	Diet				SEM	*P* value		
		SBM-TH	HSBM-TH	SBM-BP	HSBM-BP		Protein	Fiber	Protein × Fiber
Beta-hydroxybutyrate dehydrogenase	BDH1	0.636	7.93	3.82	20.3	8.9	0.19	0.19	0.64
Claudin-1	CLDN1	1.47	0.871	1.10	0.902	0.32	0.60	0.21	0.51
Gap junction protein alpha 1	GJA1	0.572	0.314	0.281	0.359	0.14	0.71	0.26	0.20
Heat shock protein 70	HSP70	0.0515	0.0637	0.275	0.143	0.071	0.44	0.61	0.44
3-Hydroxy-3-methylglutaryl-CoA synthase 2	HMGCS2	80.2	50.1	16.0	185	64	0.44	0.45	0.11
Monocarboxylate transporter isoform 1	MCT1	1.76	1.98	1.77	1.50	0.59	0.95	0.61	0.66
Monocarboxylate transporter isoform 2	MCT2	0.00552	0.0758	0.110	0.00291	0.059	0.95	0.60	**0.090**
Monocarboxylate transporter isoform 4	MCT4	0.0248	0.113	0.118	0.0264	0.072	0.83	0.86	0.20
Serine-threonine protein kinase B	AKT1	0.806	3.98	4.82	15.6	6.5	0.27	**0.088**	0.63
Sodium-hydrogen exchanger isoform 1	NHE1	0.0582	0.0921	0.0758	0.133	0.069	0.53	0.56	0.86
Sodium-hydrogen exchanger isoform 2	NHE2	1.15	1.89	1.82	4.06	0.98	0.18	**0.075**	0.42
Sodium-hydrogen exchanger isoform 3	NHE3	0.870	0.627	0.821	0.823	0.18	0.11	0.35	0.46

[1]HSBM-TH = heat-treated soybean meal and timothy hay; SBM-TH = soybean meal and timothy hay; HSBM-BP = heat-treated soybean meal and beet pulp; SBM-BP = heat-treated soybean meal and beet pulp.

Supplementary Table S1. Primers used in rumen epithelial gene expression analysis

Primer	Sequence	Accession number	Amplicon size (bp)
Beta-hydroxybutyrate dehydrogenase (BDH1)	Fwd: CCCACCACCAGTCTGAGCAT Rev: CCCACTACTCTGCACCCCAA	NM_001034600.1	101
Claudin-1 (CLDN1)	Fwd: CAGTGCAAAGTCTTCGACTCC Rev: GTCGTCTTCCATGCACTTCA	BT021861.1	147
Gap-junction protein alpha 1 (GJA1)	Fwd: ATGAGCAGTCTGCCTTTCGT Rev: AGCCAGGTACAGGAGTGTGG	NM_174068.2	143
Heat shock protein 70 (HSP70)	Fwd: AGCTGGAGCAGGTGTGTAAC Rev: AGCTTGCATAGCTGATGGCT	U09861.1	239
3-Hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2)	Fwd: TTACGGGCCCTGGACAAAT Rev: CACATCATCGAGAGTGAAAGG	NM_001045883.1	100
Monocarboxylic acid transporter 1 (MCT1)	Fwd: GTCATTGGAGGTCTTGGGCT Rev: GGTAGAGAGGAACACAGGGC	NM_001037319.1	129
Monocarboxylic acid transporter 2 (MCT2)	Fwd: TGGTCTCGGCCTCTTACAGT Rev: GCCATTCGCTACAGGTCGTT	NM_001076336.2	146
Monocarboxylic acid transporter 4 (MCT4)	Fwd: GTGACACAGCCTGGATCTCC Rev: AGAAGGACGCAGACACCATG	NM_001109980.1	150
Serine-threonine protein kinase 1 (AKT1)	Fwd: CTGCACAAGCGAGGTGAGTA Rev: GAAGTTGTTGAGGGGCGACT	NM_173986.2	132
Sodium-hydrogen exchanger 1 (NHE1)	Fwd: GTCCCACACGACCATCAAGT Rev: AGGGTGCTGATGACAAACGT	NM_174833.2	132
Sodium-hydrogen exchanger 2 (NHE2)	Fwd: CGAGCAGCTCTACATCCTGG Rev: ATGCCAGCAAACACGTCAAC	XM_604493.6	129
Sodium-hydrogen exchanger 3 (NHE3)	Fwd: CCTCATGAGAAGGTCGGCTC Rev: GAACGGATGAAAGCCAGGGA	NM_001192154.1	129
Ribosomal protein subunit 9 (RPS9)	Fwd: GTGAGGTCTGGAGGGTCAAA Rev: GGGCATTACCTTCGAACAGA	NM_001101152.2	108

Supplementary Table S2. LS means for nonsignificant VFA interconversions as differentiated by diet along with *P* values for the effects of protein source, fiber source, and the interaction of protein and fiber source[1,2]

	Diet					*P* value		
Measurement	SBM-TH	HSBM-TH	SBM-BP	HSBM-BP	SEM	Protein	Fiber	Protein × Fiber
Interconversion rate, mmol/mmol/h								
Acetate to butyrate	0.304	0.232	0.308	0.122	0.11	0.23	0.61	0.59
Acetate to valerate	0.0706	0.0303	0.0706	0.0741	0.019	0.29	0.20	0.20
Acetate to isobutyrate	0.0280	0.0291	0.0308	0.0522	0.013	0.29	0.23	0.34
Propionate to acetate	0.388	0.480	0.297	0.232	0.16	0.93	0.32	0.64
Propionate to isobutyrate	0.0222	0.0298	0.0353	0.0369	0.013	0.71	0.42	0.81
Butyrate to valerate	0.103	0.0231	0.0362	0.0444	0.035	0.26	0.47	0.18
Butyrate to isovalerate	0.0169	0.0182	0.0162	0.0301	0.011	0.45	0.58	0.53
Butyrate to isobutyrate	0.0106	0.0163	0.0161	0.0330	0.0088	0.19	0.20	0.50
Valerate to propionate	0.293	0.325	0.390	0.357	0.12	0.99	0.57	0.77
Valerate to butyrate	0.0666	0.0876	0.0656	0.0632	0.040	0.77	0.70	0.72
Valerate to isobutyrate	0.0441	0.0992	0.0779	0.0802	0.042	0.48	0.85	0.51
Isovalerate to acetate	0.249	0.327	0.312	0.209	0.099	0.88	0.75	0.30
Isovalerate to propionate	0.313	0.204	0.225	0.222	0.083	0.51	0.67	0.53
Isovalerate to butyrate	0.200	0.376	0.254	0.404	0.11	0.17	0.71	0.91
Isovalerate to valerate	0.0532	0.0602	0.151	0.135	0.071	0.95	0.25	0.87
Isovalerate to isobutyrate	0.0308	0.0683	0.0440	0.0535	0.018	0.18	0.96	0.40
Isobutyrate to acetate	0.198	0.207	0.176	0.276	0.11	0.64	0.84	0.69
Isobutyrate to propionate	0.149	0.176	0.298	0.203	0.10	0.69	0.32	0.48
Isobutyrate to valerate	0.0954	0.121	0.0694	0.134	0.069	0.48	0.92	0.76
Isobutyrate to isovalerate	0.0362	0.0486	0.0715	0.0679	0.025	0.83	0.20	0.69
Interconversion flux, mmol/h								
Acetate to isobutyrate	0.478	0.458	0.538	1.12	0.30	0.19	0.10	0.16
Propionate to acetate	4.44	4.17	1.58	2.27	1.9	0.91	0.22	0.80
Propionate to butyrate	2.50	3.50	1.18	1.53	0.99	0.50	0.12	0.74
Propionate to valerate	0.809	0.811	0.349	0.682	0.34	0.58	0.34	0.59
Propionate to isobutyrate	0.272	0.210	0.339	0.469	0.17	0.78	0.20	0.43
Butyrate to acetate	1.76	1.93	1.46	2.17	0.68	0.43	0.95	0.63
Butyrate to propionate	1.72	1.57	2.00	1.95	0.74	0.86	0.56	0.93
Butyrate to valerate	0.278	0.190	0.172	0.645	0.18	0.31	0.35	0.15
Butyrate to isovalerate	0.0476	0.118	0.157	0.601	0.20	0.19	0.14	0.33
Butyrate to isobutyrate	0.0471	0.128	0.115	0.515	0.20	0.22	0.25	0.41
Valerate to acetate	0.608	0.320	0.259	0.964	0.33	0.54	0.66	0.16
Valerate to propionate	0.352	0.510	0.400	0.799	0.30	0.35	0.56	0.68
Valerate to butyrate	0.0465	0.115	0.0532	0.1195	0.056	0.17	0.90	0.98
Valerate to isobutyrate	0.0286	0.152	0.0836	0.188	0.080	0.16	0.55	0.91
Isovalerate to acetate	0.111	0.211	0.424	0.251	0.15	0.81	0.26	0.38
Isovalerate to propionate	0.142	0.163	0.325	0.377	0.18	0.81	0.21	0.92
Isovalerate to butyrate	0.0892	0.323	0.318	0.569	0.18	0.12	0.12	0.95
Isovalerate to valerate	0.0233	0.0632	0.129	0.587	0.27	0.37	0.26	0.45
Isovalerate to isobutyrate	0.0174	0.0692	0.0502	0.105	0.036	0.13	0.32	0.97
Isobutyrate to acetate	0.0873	0.0891	0.111	0.334	0.11	0.34	0.26	0.35
Isobutyrate to valerate	0.0856	0.104	0.0483	0.245	0.12	0.34	0.64	0.43

[1]HSBM-TH = heat-treated soybean meal and timothy hay; SBM-TH = soybean meal and timothy hay; HSBM-BP = heat-treated soybean meal and beet pulp; SBM-BP = heat-treated soybean meal and beet pulp.
[2]mmol/mmol/h = fraction of carbon per hour.

CHAPTER 5: Variation in Animal Performance Explained by the Rumen Microbiome or by Diet Composition

This chapter is published as: Gleason, C. B., and R. R. White, 2018. Variation in animal performance explained by the rumen microbiome or by diet composition. Journal of Animal Science, 96:4658-4673.

ABSTRACT

The central aim of this meta-analysis was to determine if the rumen microbiome can serve as an accurate predictor of performance in beef and dairy cattle compared with predictions based on diet composition. To support this comparison, a set of models was derived and compared. Models predicted milk yield (**MY**), average daily gain (**ADG**), dry matter intake (**DMI**), dairy feed efficiency (**FE**), and beef FE using different sets of independent variables: diet (**D**), microbial (**M**), and experimental (**E**). Diet independent variables included dry matter (**DM**), organic matter, neutral detergent fiber, acid detergent fiber, crude protein, ether extract, non-fiber carbohydrate, starch, and forage percentages. Microbiome variables included relative abundance of 3 major rumen bacterial phyla, species richness, and species diversity. Experimental variables included publication year, breed type (dairy, beef, or *Bos indicus*), and rumen sampling fraction (fluid or solid). A second set of models used D and E variables as predictors for the microbiome. For both the production and microbiome model sets, predictor variable sets were used individually and in combination. Linear mixed-effects regression, weighted by 1/standard error, was used to derive models using data from 51 peer-reviewed publications. Models for the same response variable were compared on the basis of concordance correlation coefficient with study effects removed (**uCCC**), root estimated variance associated with study and error, and corrected Akaike information criterion values, where appropriate. The MY model using D + M + E predictors outperformed all other MY models (uCCC = 0.71). Average daily gain was most accurately predicted by D alone (uCCC = 0.92). Interestingly, M + E was more successful at predicting DMI than any model using D variables. Similarly, dairy FE was more accurately predicted by M + E than D, albeit only slightly (uCCC = 0.69 vs. 0.65). Beef FE could only be modeled using D variables. Overall, breed type proved a better predictor of relative abundances

of most rumen bacterial phyla than D. Conversely, species richness and diversity indicators were unaffected by breed type but could be predicted by D with moderate precision and accuracy (uCCC = 0.63 to 0.69). This analysis suggests that diet and the microbiome may exert independent effects on various aspects of performance. Further research is necessary to determine the reasons for these independent influences.

Keywords: growth, meta-analysis, microbiome, milk production

INTRODUCTION

The rumen microbial community has long been a target of scientific interest because it is known to play a major role in feed digestion and nutrient uptake by the host (Hungate et al., 1964; Krause et al., 2013). Rumen microbes allow ruminants to harvest energy in the form of volatile fatty acids (**VFA**) through fermentation of otherwise indigestible plant material. This energy represents approximately 70% of the metabolic energy utilized by the animal (Bergman, 1990). In this way, the microbiome may serve as a connection between diet and host productivity (Mullins et al., 2013). Although much investigative work has been done on dietary influences of the microbiome, the link between microbiome and production traits in beef and dairy cattle remains unclear (Myer et al., 2015; Shabat et al., 2016; Indugu et al., 2017). By assembling the available literature into a meta-analysis, a better understanding of the microbial factors affecting animal performance may be realized. Therefore, the first objective of this meta-analysis was to determine if the rumen microbiome can serve as an accurate predictor of animal performance and compare its predictive power to that of diet, intake, and experimental explanatory variables. The second objective was to evaluate the ability of diet variables to alter microbiome characteristics. We hypothesized that 1) animal performance can be predicted by models using microbiome variables with accuracy similar to diet variables and 2) variation in the microbiome can be accurately modeled using diet variables.

MATERIALS AND METHODS

Data Collection and Preparation

Published, peer reviewed journal articles provided the data used in this meta-analysis. To be selected, papers were required to report relative abundances of bacterial phyla in rumen

microbial samples taken from dairy or beef cattle. *Bos taurus* and *Bos indicus* breeds were accepted into the dataset. Evaluation of the microbial community must have been conducted using 16S rRNA sequencing. As a final search criterion, papers needed to include a list of diet ingredients and their inclusion rates. Preferably, studies also utilized multiple diet treatments, provided complete chemical composition of the diets, and measured milk yield (**MY**) or average daily gain (**ADG**) in dairy and beef cattle, respectively. The literature search was conducted using the Google Scholar search engine (https://scholar.google.com) from September 1 to November 25, 2017 and included any paper meeting the above criteria, regardless of publication date. Keywords used to obtain papers were as follows: "cattle," "beef," "dairy," "16s rRNA sequencing," "microbe," "microbiome," "bacteria," "diet," "rumen," "production," "milk yield," and "average daily gain". Relevant citations from the reference sections of each selected article were also considered for inclusion. Fifty-one publications with a total of 230 observations were ultimately obtained. All publications are listed in Supplementary Table S1 and a summary of the data is presented in Table 1. In cases where relevant information was not reported in the paper, an inquiry was sent to the corresponding author to obtain those data, if possible. In cases where dietary chemical composition was not reported and could not be obtained from the corresponding author, it was calculated using average nutrient values for individual feed ingredients sourced from the Dairy One Feed Composition Library (http://dairyone.com/), Feedipedia (https://www.feedipedia.org), or online product information for certain commercial feed supplements.

Derivation of Models

All data were analyzed using R version 3.4.1 (R Core Team, 2017). The R package lme4 version 3.4.3 was used for model derivation (Bates et al., 2015). Models were divided into 2

major sets. Set 1 included the production response variables dry matter intake (**DMI**), MY, ADG, dairy feed efficiency (**FE**), and beef FE. Dairy FE was calculated as MY/DMI and beef FE was calculated as ADG/DMI. Set 2 was composed of models predicting Bacteroidetes relative abundance, Firmicutes relative abundance, Bacteroidetes-Firmicutes ratio (**B:F**), Proteobacteria relative abundance, Chao1 richness, and Shannon diversity. For Set 1 models, explanatory variables used were classified as diet, microbial, or experimental method variables. Diet variables included dry matter (**DM**), organic matter (**OM**), crude protein (**CP**), neutral detergent fiber (**NDF**), acid detergent fiber (**ADF**), nonfiber carbohydrate (**NFC**), starch, ether extract (**EE**), and dietary forage percentage. All were expressed as percentages on a DM basis except DM, which was expressed as a percentage on an as-fed basis. Microbial variables were represented by 3 major bacterial phyla: Bacteroidetes, Firmicutes, and Proteobacteria, and B:F. Two other microbial variables were Chao1 richness, an estimate of species richness, and Shannon diversity, an indication of species abundance and diversity. The experimental variables set included publication year, breed type (dairy, beef, or *B. indicus*), and rumen sampling fraction (fluid or solid). These 3 explanatory variable sets were utilized separately and in combination in order to generate multiple models for comparison and, most importantly, to examine the predictive power of microbe-based models relative to diet-based ones. An additional model for MY and ADG was derived using microbiome variables plus DMI only. Explanatory variable classes for Model Set 2 included diet variables, experimental variables, and both variable types in combination. The experimental variables used in a particular model depended on what was relevant for the response variable under consideration and could include year of publication, breed type (dairy, beef, or *Bos indicus*), or rumen sampling fraction (fluid, solid, or mixed). For example, rumen sampling fraction was not included in any model that did not also

contain microbial variables, and breed type was not used in any MY model because dairy type was the only breed type for which MY data were available.

Models were derived using linear mixed-effects regression with a random study effect. Error normality was checked at each step of the model derivation process through evaluation of residual plots. Weights used in regressions to derive models for MY and ADG were calculated as 1/standard error of the mean (SEM). Regressions to derive models for dairy FE and beef FE utilized the same weight as MY and ADG, respectively. All other regressions were unweighted due to a paucity of reported SE values for microbiome variables. The majority of studies reported SEM values. When not reported, the corresponding author was able to supply the SEM estimates or provide raw data from which they could be calculated.

Most models were generated using a backward elimination approach following the approach described in Roman-Garcia et al. (2016) and White et al. (2016). Briefly, all variables within 1 or more explanatory variable sets were included in an initial model derivation, and variables were removed from the model one by one in order of least significance ($P > 0.10$). When variables within a model were reduced to only those with a significant effect ($P \leq 0.05$) or that exhibited a trend towards significance ($0.05 < P \leq 0.10$), variables that had previously been eliminated were reintroduced and evaluated for significant effects in reverse order of removal. When 2 or more models using variables from the same explanatory variable set(s) had been generated but could not be combined without losing significance, the model with the lowest small-sample-size corrected version of Akaike information criterion (**AICc**) was selected as the final model (Hurvich and Tsai, 1989). In cases where the number of observations for a response variable was too few to generate a model containing all candidate explanatory variables within a set, a forward selection approach was used where variables were introduced one at a time in

order of highest number of observations and evaluated for significant effects. For instances where 2 variable sets were used for model derivation but no variables from 1 set showed significance or a trend towards significance, the variable with the lowest *P* value was retained to show the lack of significance of all variables in the set. Any variable contained within a significant interaction was retained, regardless if it demonstrated a significant main effect. After derivation by the above procedure, a model's variables were evaluated for multicollinearity using variance inflation factors (**VIF**) as described by Akinwande et al. (2015). Variables were required to have a VIF < 10 to remain in the model, but variables with a VIF between 10 and 100 were retained if they were part of an interaction (Roman-Garcia et al., 2016). Importantly, VIF was not used as a model selection criterion; rather, it was used to affirm that final models did not suffer from multicollinearity. Slope-by-study interactions were tested as part of final model confirmation. Significant slope-by-study interactions are indicated in the model tables. In cases where slope-by-study interactions were present, coefficients should not be considered reliable and consistent cross-study relationships and model performance in an external dataset should be assumed to be poor.

Evaluation of Models

Model quality was assessed on the basis of the standard deviation for study ($\hat{\sigma}_s$), as well as the standard deviation for error ($\hat{\sigma}_e$). The ratio of $\hat{\sigma}_s$ to $\hat{\sigma}_e$ was also calculated. In cases where $\hat{\sigma}_s$ is much greater than $\hat{\sigma}_e$, the model may be acceptable on average but would not be expected to predict new, single observations well. Root mean square error (**RMSE**) was not considered in model evaluation since a random study effect was used. According to Boerman et al. (2015), the use of RMSE to select models in this situation results in models that are unable to effectively account for error with regards to future data. The AICc was used to evaluate the relative quality

of the models when derived against identical datasets. Finally, Lin's concordance correlation coefficient (**CCC**) was used to evaluate agreement between variables (Lin, 1989). Coefficients were obtained both as adjusted for study (**CCC**) or those in which a study-specific calibration was assumed to be unavailable (**uCCC**). For final comparisons of models using different derivation datasets, uCCC was the main indicator of model superiority. As explained by White et al. (2017), the use of uCCC is an imperfect but preferable strategy to comparison using CCC because it provides a more reliable indication of model fit in situations where calibration for local (study) conditions is not possible before model use. Models were tested for competency at predicting new observations using Monte Carlo cross-validation.

RESULTS AND DISCUSSION

Description of Data

Descriptive statistics for the data are presented in Table 1. Tables S2 and S3 summarize the variables used in successful model derivation of animal performance measurements and microbiome characteristics, respectively. Supplementary Table S4 lists our assumptions for nutrient composition of select diet ingredients. Parameter estimates for models along with fit statistics can be found in Tables 2 through 12. Cross-validation results are presented in Supplementary Tables S5 to S13. Average daily gain and beef FE models, along with DMI Model 7, dairy FE Models 1, 5, and 6, and Chao1 richness Models 2 and 3, could not be cross-validated due to an insufficient number of observations to generate reliable training and test datasets.

Prediction of Animal Performance

Milk yield. Of the 8 models intended for prediction of MY (Table 2), the 3 explanatory variable sets individually and in combination resulted in successful derivation of 5 unique models where all variables significantly contributed, or tended to contribute, to the prediction. Model 8, generated using D + M + E predictors, outperformed all other MY models (uCCC = 0.71; Table 2). Cross-validation confirmed the superiority of Model 8 over the others (uCCC = 0.81; Supplementary Table S5), indicating that it would be expected to perform well given a new set of observations. Including DMI with M predictor variables resulted in a better fit than M alone (uCCC = 0.58 vs. 0.49, Table 2), making Model 3 the second-best model. This improvement in model fit was anticipated because higher levels of feed intake support greater levels of milk production. Close agreement was also observed after Model 3 was cross-validated (uCCC = 0.57; Supplementary Table S5). Aside from DMI, CP was the only diet-related variable that proved significant ($P < 0.01$; Table 2) for MY predictions. Although CP has consistently been shown to relate positively with MY (Van Horn et al., 1979; Kalscheur et al., 1999; Law et al., 2009), it is somewhat surprising that other dietary descriptors (starch, NFC, EE, etc.) did not significantly influence MY. Model 6 included NFC, but only as a trend ($P = 0.058$, Table 2). The lack of clear significance could be due to the nonspecificity of NFC as a nutrient and is supported by previous work identifying that varying levels of NFC did not significantly impact MY (Batajoo and Shaver, 1994).

Relative abundances of Bacteroidetes and Firmicutes, and B:F were the major microbial predictors of MY. This is fairly unsurprising as these phyla generally comprise the vast majority of the microbiome and would thus be expected to exert a measurable influence on the host. The identified relationships between microbial parameters and MY are consistent with the literature.

Indugu et al. (2017) reported that a number of Bacteroidetes taxa were positively correlated with MY, and most Firmicutes taxa were associated with higher levels of milk fat and milk protein and lower MY. Jami et al. (2014) also identified a Firmicutes family that was negatively correlated with MY. Proteobacteria remained significant in Model 2 ($P = 0.02$; Table 2) and Chao1 richness remained significant in Models 2 ($P = 0.0048$) and 7 ($P = 0.0091$), but these models had weaker predictive power compared with the other successfully derived MY models (uCCC = 0.49 and 0.30, respectively). Proteobacteria has been shown to correlate with MY, both negatively and positively (Indugu et al., 2017), likely indicating that specific taxa within this phylum have diverse metabolic functions which can result in different levels of host performance.

Dairy feed efficiency. Derivation yielded 4 successful models for the prediction of dairy FE, which can be found in Table 3. Compared with beef FE, determining FE in dairy cows is considerably more challenging since cows' bodies may be undergoing tissue accretion or mobilization during lactation. Interpretation of the dairy FE models presented here must therefore include the understanding that tissue turnover is also occurring even though it cannot be accounted for in the models. With the exception of Shannon diversity, the majority of the variables that remained significant predictors were diet variables. Overall, Model 5 (D + E variables) resulted in the best prediction for dairy FE (uCCC = 0.88; Table 3). Interestingly, the inclusion of publication year, while not significant ($P = 0.073$), improved fit over Model 1 (D variables; uCCC = 0.65; Table 3). Although the inclusion of year as a variable can be detrimental due to its confounding effect, it may be expected to encompass the gradual improvement in obtaining and handling microbial data, as well as other variables that change with time that could not be accounted for. When rumen sampling type was accounted for,

microbiome resulted in a similar but marginally improved prediction compared with the model containing only D variables (uCCC = 0.69 vs. 0.65; Table 3). Not accounting for sampling type resulted in a decidedly poorer model (uCCC = 0.26; Table 3).

Consistent with previous findings, NDF and ADF were associated with decreased FE (Britt et al., 2003; Broderick, 2003). Higher levels of fiber result in lower digestibility and decreased energy density of a diet, which in turn would negatively impact FE. Broderick (2003) reported that greater dietary energy density improved FE by supporting a higher rate of milk production without increasing DMI. It was also reported that increased CP content led to a reduction in FE, which was reflected in our models ($P < 0.001$; Table 3). Broderick (2003) found that increasing CP from 16.7% to 18.4% resulted in greater DMI while milk production remained unchanged, causing FE to decline. Although CP has been shown to relate positively with MY (Van Horn et al., 1979; Kalscheur et al., 1999; Law et al., 2009), higher levels of CP appear to be accompanied by diminishing returns in terms of efficiency to the point that FE begins to suffer. Ether extract also proved to be a significant ($P < 0.001$; Table 3) predictor variable and was negatively associated with dairy FE. Increasing levels of dietary fat have been shown to positively affect DMI (Allen, 2000). The effects of EE on MY have been less consistent in the literature, but meta-analysis work has revealed a decline in MY at high levels of fat supplementation (Patra, 2013). Increases in dietary fat may impede fiber digestion and exert a toxic effect on certain rumen microbes. Efficiency would therefore be expected to decrease as EE levels increase.

Shannon diversity was a significant ($P < 0.05$; Table 3) microbiome variable for the prediction of dairy FE. The significance of this diversity metric agrees with findings of increased energy harvesting capacity in ruminants as well as humans (Turnbaugh et al., 2009; Shabat et al.,

2016). The microbiomes of inefficient cows have been characterized as more diverse from the standpoint of taxa and are found to employ a higher number of metabolic pathways than the microbiomes of efficient cows. Conversely, efficient cows' microbial communities generally have more dominant taxa and rely on a smaller number of metabolic pathways that are more energetically valuable to the animal (Shabat et al., 2016). Diversity therefore appears to be negatively associated with a microbiome's ability to supply its host with the energy needed for production.

Average daily gain. Four models predicting ADG were generated (Table 4). Variables used in successful derivation of these models were similar to those used for the prediction of MY. For ADG Model 1 (D variables), DMI increased performance ($P < 0.001$; Table 4). This effect is logical because a higher level of intake by an animal would be expected to increase the rate of weight gain under normal circumstances. Nonfiber carbohydrate also remained significant in ADG Model 1 ($P < 0.001$; Table 4). Starches and sugars represent the predominant fermentative fractions of concentrate diets, which support rapid growth compared to forage diets (Whiting, 1957). Furthermore, forage was included in Model 5 (D + M; $P = 0.0054$; Table 4) and was negatively associated with ADG.

Significance of microbial variables in the ADG models was less apparent than in MY models, with B:F being the only significant variable from this set ($P = 0.028$; Model 2; Table 4). The Bacteroidetes-Firmicutes ratio has been suggested to affect energy harvesting ability and body fatness in mice and humans (Turnbaugh et al., 2006), with a lower ratio favoring efficient energy uptake and greater fat accretion. Our model was consistent with these findings, indicating that a lower B:F supports a higher rate of gain in finishing steers. In Model 7 (M + E), accounting for breed type resulted in an improved fit over Model 2 (M variables), which would

be anticipated since *B. indicus* cattle have a slower rate of growth compared with *B. taurus* breeds. On the basis of fit statistics, the microbe model had slightly lower predictive ability (uCCC = 0.88; Table 4) compared with the remaining models. Model 1 (D variables), Model 5 (D + M), and Model 7 (M + E) all possessed a similar fit (uCCC = 0.92; Table 4). All 4 models had the same number of observations (n = 10). Model 1, however, possessed the lowest error estimate ($\hat{\sigma}_e$ = 0.03; Table 4). Diet alone, therefore, was deemed the best predictor of ADG; however, much of the variance associated with diet could also be explained by variance in the microbiome.

Beef feed efficiency. Due to an insufficient number of observations, most models for the prediction of beef FE could not be derived. No microbe variables were included as significant in the models; the lowest P value for a microbe variable was P = 0.77 for B:F (Table 5). The only successful model that could be derived was Model 1, shown in Table 5, using the diet variables starch and EE. The inclusion of starch increases the energy density of a diet and would result in greater efficiency by increasing the rate of gain without increasing intake. Supplementation of fat to increase diet energy density has also been demonstrated to improve FE (Brandt and Anderson, 1990). Krehbiel et al (1995), for instance, reported an average improvement in FE of 4.4% when finishing steers received tallow as part of their ration. The fit of this model was virtually perfect (uCCC > 0.99; Table 5); however, this may be attributed in part to the small number of observations that the models were able to utilize (n = 10).

Dry matter intake. Six models for the prediction of DMI are presented in Table 6. Model 7 (D + M + E) was unsuccessful due to insignificance of diet variables. Model 6 (M + E) possessed the best fit compared with the others (uCCC = 0.93; Table 6) and was also the best-performing DMI model when cross-validated (uCCC = 0.86; Supplementary Table S7). However, Model 2, using

only the M variable set, performed poorly compared with the remaining DMI models (uCCC = 0.46; Table 6). The DMI prediction using D variables also benefited slightly when E variables were accounted for (uCCC = 0.69 vs. 0.76; Table 6). In this way, the E variables set appeared with more prominence in DMI predictions than in the other production model sets by greatly improving model fits. Inclusion of the breed type variable was especially important due to large intake differences between breed types. Unsurprisingly, dairy cattle had a significantly higher level of DMI compared to beef type breeds ($P < 0.05$; Table 6), while *B. indicus* cattle tended to consume less than *B. taurus* beef breeds.

Starch, NFC, and forage were significant diet variables for our intake predictions ($P < 0.05$; Table 6). Dietary starch content has been implicated in some cases as an intake reducer, which is consistent with our findings. Fermentation of starch in the rumen results in propionate production which, when absorbed, has been shown to exert a hypophagic effect in numerous studies (Anil and Forbes, 1980; Elliot et al., 1985; Farningham and Whyte, 1993; Sheperd and Combs, 1998). Nonfiber carbohydrate content, conversely, was shown to positively affect DMI in our predictions. Previous work has shown that increasing levels of dietary sugar content stimulate greater intake (Broderick et al., 2008; Penner and Oba, 2009). Broderick et al. (2008), for instance, reported linear increases in DMI as cornstarch was replaced with sucrose. Because starch is included within NFC, the work of Broderick et al. (2008) suggests that the sugar fraction of NFC is primarily responsible for stimulating DMI. As would be expected, dietary forage content was a significant predictor of DMI ($P = 0.014$ for D model; $P = 0.005$ for D + E model; Table 6) and was associated with decreased intake. Depressed DMI is a frequent observation as the forage-to-concentrate ratio of a diet increases (Colucci et al., 1982; Sheperd and Combs, 1998).

Microbial variables appearing in our DMI predictions were B:F and Shannon diversity. Shannon diversity was significant ($P = 0.022$; Table 6) in the D + M + E model and tended ($P = 0.099$; Table 6) to contribute to the prediction in the M model, whereas B:F tended ($P = 0.080$; Table 6) to contribute to the prediction in the D + M model. Because these variables appeared frequently in other models (MY, dairy FE, and ADG), it appears that they may play especially important roles concerning nutrient metabolism in the rumen and energy availability to the host.

Necessity of Accounting for Sample Type

In addition to ensuring that potential differences in breed type were identified, the E variable set was mainly included to determine if the predictive power of microbial variables was impacted by the rumen sampling fraction from which they were obtained. Samples taken from rumen fluid or solids can yield significantly different microbial profiles because these 2 fractions represent distinctly different ecological niches within the microbiome (McAllister et al., 1993; Klevenhusen et al., 2017). Klevenhusen et al. (2017), for example, noted that the most abundant phylum found in the rumen fluid was Firmicutes while the bacteria associated with the solid fraction were predominantly Bacteroidetes. Accounting for sampling type often benefited model predictive power, notably for MY Model 8, dairy FE Model 6, and DMI Model 6.

Prediction of the Microbiome

Derivation of models predicting the microbiome yielded several that appeared promising. In a cross-validation setting, however, the microbiome models performed poorly (Supplementary Tables S8 to S13). This indicates that models would lack competency when used to predict new data. Given the substantial between-study variation in the microbiome literature (inconsistent identification of genera, varying bioinformatics methods, etc.), the poor predictive performance

of these models is not surprising. This expected poor predictive performance is, in part, why the objectives of this study centered on explaining variation in the available data, rather than generating models that are expected to generate robust predictions of microbial data. Throughout the discussion of these findings, emphasis will therefore be placed on causes of microbial variation rather than on the predictability of the microbiome.

Relative abundances of phyla. In spite of the massive body of research in existence detailing the effects exerted by diet on the rumen microbiome, diet variables were unable to predict the relative abundances of Bacteroidetes or Firmicutes, or B:F for our model sets (Tables 7 to 9). This surprising observation may be due to the fact that a phylum will contain many species that possess diverse substrate preferences and activities. Inconsistencies have also been noted in the literature. AlZahal et al. (2017) noted that, although increases in the genus *Bacteroidales* were observed on high-concentrate diets, Henderson et al. (2015) reported higher abundances of *Bacteroidales* on low-concentrate diets. It is therefore possible that we did not see significance of certain diet variables due to these inconsistencies within the literature.

Although diet could not be used to predict Bacteroidetes or Firmicutes abundances or B:F, there were significant differences ($P < 0.05$) between breed types, as shown in Tables 7 through 9. *B. indicus* cattle had significantly higher abundances of Bacteroidetes ($P < 0.05$; Table 7) and significantly lower abundances of Firmicutes ($P < 0.05$; Table 8) compared to *B. taurus* beef breeds. This was also observed for B:F, where a *B. indicus* breed type was associated with a significantly higher ratio than *B. taurus* beef breeds ($P < 0.05$; Table 9). Abundances for Bacteroidetes and Firmicutes and B:F were not significantly different ($P > 0.05$; Tables 7 to 9) between dairy-type cattle and *B. taurus* beef breeds. As previously discussed, B:F may be a significant determinant of an individual's energy harvesting ability (Turnbaugh et al., 2006). As

B. taurus breeds have undergone intensive selection for production and efficiency compared with *B. indicus* cattle, our results appear to align with this theory. To the best of our knowledge, this particular distinction between *B. taurus* and *B. indicus* microbiomes has not been thoroughly investigated.

Unlike the other phyla examined in this meta-analysis, the relative abundance of Proteobacteria could be modeled using diet variables. As shown in Table 10, Model 1 included EE and forage as significant predictors ($P < 0.001$ and $P = 0.023$, respectively). Model 2 indicated only a tendency for *B. indicus* breed type to differ from *B. taurus* beef cattle in relative abundance. Both models possessed a fairly similar fit with Model 1 having a slight advantage (Model 1 uCCC = 0.69 vs. Model 2 uCCC = 0.67).

The effect of EE on Proteobacteria is consistent with previous findings in murine models that reported a significant increase in the relative abundance of gut Proteobacteria, or specific taxa belonging thereto, when dietary fat levels were increased (Hildebrandt et al., 2009; de La Serre et al., 2010; Devkota et al., 2012). Hildebrandt et al. (2009) attributed the increase in Proteobacteria partly to upregulation of bacterial genes associated with movement, specifically chemotaxis and flagellar function, which were stimulated by high fat intake. It is therefore possible that members of this phylum gained an advantage due to enhanced motility. The level of forage vs. concentrate has also been demonstrated to impact the relative abundance of Proteobacteria in cattle (Auffret et al., 2017). Although we also identified a relationship between forage and Proteobacteria in this meta-analysis, the directionality of the relationship differs from that reported by Auffret et al. (2017), suggesting further work may be needed to understand how Proteobacteria respond to forage concentrations.

Chao1 richness and Shannon diversity. Richness and diversity indices are commonly used by microbiologists to gain valuable insight into a bacterial community. As an estimator of species richness, the Chao1 index provides a prediction for the number of species in a given microbial sample whereas Shannon diversity provides an indication of species representation within a sample by taking both sample richness and the sample's evenness of taxa into account.

Models for Chao1 richness and Shannon diversity, shown in Tables 11 and 12, respectively, were impacted by diet variables but were not sensitive to breed type. Diet-induced changes in these indices have been observed, particularly when animals are shifted from high-forage to high-concentrate diets (Mao et al., 2013; AlZahal et al., 2017; Zhang et al., 2017). In our models, OM was significant ($P < 0.001$; Table 11) for the prediction of Chao1 richness whereas CP and starch tended to contribute to the prediction ($0.05 < P \leq 0.10$; Table 11). This indicates that the total number of bacterial species within the microbiome is likely affected by specific dietary components because species may grow or decline in numbers depending on the availability of their preferred substrate. For example, NDF was significant ($P < 0.001$; Table 12) for the prediction of Shannon diversity. A higher NDF percent, indicative of greater dietary forage content, has been observed to coincide with a higher degree of diversity compared with high-concentrate diets (AlZahal et al., 2017). A decrease in fiber levels appears to lower diversity by reducing substrate variety for the microbiome and by allowing rumen pH to drop, prompting certain taxa to become more dominant while others recede.

Limitations and Future Directions

As shown in Tables 4 and 5, 1 model for ADG and 5 models for beef FE could not be derived due to insufficient observations. Numbers of observations for the remaining models were

still small, ranging from 6 to 22. This lack of sufficient data most likely contributed to the inability of microbiome variables to remain in most of the predictions for beef performance and underscores the need for further investigation of the beef microbiome. Without more data, it will be difficult to improve our understanding of microbial influences on production traits in beef cattle. Additionally, future meta-analyses could focus on the link between the microbiome and quality of production in beef and dairy cattle, rather than quantity, examining traits such as marbling and milk fat.

Another potential challenge to model accuracy is the fact that this analysis relied on examination of group means rather than data from individual animals. It has been shown that the microbiome may vary considerably between hosts (Weimer et al., 2010; Jami and Mizrahi, 2012). For instance, Jami and Mizrahi (2012) noted that approximately 50% of detected bacterial species were present in only 30% or less of the animals studied, regardless of identical diets and experimental conditions. Further analysis, however, revealed 82% phylogenetic similarity between their samples. It was concluded that certain species may differ between individual microbiomes but their genetic relatedness makes it possible for them to function in similar capacities and niches. It is therefore possible that animal-dependent microbial variation was less of an issue in our analysis because we examined microbial relative abundances on the basis of phylum rather than genus.

Although 16S rRNA gene sequencing has allowed for deeper investigation of bacterial communities like the rumen microbiome, its ability to accurately represent such communities can be impacted by a number of factors. Differences in community composition may be observed depending on the particular region of the 16S gene that is chosen for amplification and sequencing (Tremblay et al., 2015). To date, there is little agreement on which region provides

the most dependable profile. The PCR amplification step is known to introduce corrupt sequences such as chimeras, which are sequences generated from the combination of 2 or more parent sequences (Haas et al., 2011). The presence of chimeras can lead to an overestimation of richness because they are often treated as legitimate sequences. There also appears to be some variation between different sequencing platforms, but the impacts of this variation on accuracy have been deemed relatively minor (Tremblay et al., 2015). Overall, these limitations can create difficulty in classifying bacteria to the level of genus or species (Boudewijns et al., 2006). Classification errors at these levels have the potential to influence richness and diversity estimates. Despite the above criticisms of 16S rRNA sequencing, this analysis was able to use data from mostly 16S rRNA studies in successful predictions of animal performance. Our results therefore indicate 16S rRNA sequencing may be sufficiently dependable for adequate characterization of the rumen microbiome.

Further examination of the microbiome from a functional rather than a taxonomic standpoint is yet another necessary angle of investigation. Although certain bacteria may share genetic similarities, their functions and subsequent impacts on the host can be very different (Jewell et al., 2015; Pitta et al., 2016). Performance predictions using the microbiome might therefore be improved by focusing on overall and specific metabolic activities of the microbes rather than population estimates. Metatranscriptomics has demonstrated effectiveness as a tool to characterize the metabolic activities of the microbiome based on the identification of prominent enzymatic pools and pathways (Helbling et al., 2012). Research by Li and Guan (2017), which utilized metatranscriptomics to demonstrate links between the microbiome and beef FE, provides an example of the type of future work that will improve our understanding of microbial factors influencing production traits in ruminants.

Conclusions

This meta-analysis sought to explore the connections among microbiome, diet, and animal performance and, in doing so, provide valuable findings for the fields of microbial ecology and production management. In a major way, the microbiome serves as the intermediate between diet and performance because the majority of a ruminant's fuel supply is generated by microbial fermentation of feedstuffs. Changes in the microbiome would therefore be expected to influence production traits. Our results indicate that the microbiome may serve, in some cases, as a more accurate predictor of animal performance than diet alone or enhance predictions in combination with diet. Further study of the complex interrelationships among diet, microbiome, and host is necessary to improve efforts in optimizing performance in ruminant animals.

LITERATURE CITED

Akinwande, M. O., H. G. Dikko, and A. Samson. 2015. Variance inflation factor: As a condition for the inclusion of suppressor variable(s) in regression analysis. Open J. Stat. 05:754–767. doi:10.4236/ojs.2015.57075.

Allen, M. S. 2000. Effects of diet on short-term regulation of feed intake by lactating dairy cattle. J. Dairy Sci. 83:1598–1624. doi:10.3168/jds.S0022-0302(00)75030-2.

AlZahal, O., F. Li, L. L. Guan, N. D. Walker, and B. W. McBride. 2017. Factors influencing ruminal bacterial community diversity and composition and microbial fibrolytic enzyme abundance in lactating dairy cows with a focus on the role of active dry yeast. J. Dairy Sci. 100:4377–4393. doi:10.3168/jds.2016-11473.

Anil, M. H., and J. M. Forbes. 1980. Feeding in sheep during intraportal infusions of short-chain fatty acids and the effect of liver denervation. J. Physiol. 298:407–414. doi:10.1113/jphysiol.1980.sp013090.

Auffret, M. D., R. J. Dewhurst, C.-A. Duthie, J. A. Rooke, R. John Wallace, T. C. Freeman, R. Stewart, M. Watson, and R. Roehe. 2017. The rumen microbiome as a reservoir of antimicrobial resistance and pathogenicity genes is directly affected by diet in beef cattle. Microbiome. 5:159. doi:10.1186/s40168-017-0378-z.

Batajoo, K. K., and R. D. Shaver. 1994. Impact of nonfiber carbohydrate on intake, digestion, and milk production by dairy cows. J. Dairy Sci. 77:1580–1588. doi:10.3168/jds.S0022-0302(94)77100-9.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67:1–48. doi:10.18637/jss.v067.i01.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70:567–590. doi:10.1152/physrev.1990.70.2.567.

Boerman, J. P., J. L. Firkins, N. R. St-Pierre, and A. L. Lock. 2015. Intestinal digestibility of long-chain fatty acids in lactating dairy cows: A meta-analysis and meta-regression. J. Dairy Sci. 98:8889–8903. doi:10.3168/jds.2015-9592.

Boudewijns, M., J. M. Bakkers, P. D. J. Sturm, and W. J. G. Melchers. 2006. 16S rRNA gene sequencing and the routine clinical microbiology laboratory: A perfect marriage? J. Clin. Microbiol. 44:3469–3470. doi:10.1128/JCM.01017-06.

Brandt, R. T., and S. J. Anderson. 1990. Supplemental fat source affects feedlot performance and carcass traits of finishing yearling steers and estimated diet net energy value. J. Anim. Sci. 68:2208–2216. doi:10.2527/1990.6882208x.

Britt, J. S., R. C. Thomas, N. C. Speer, and M. B. Hall. 2003. Efficiency of converting nutrient dry matter to milk in Holstein herds. J. Dairy Sci. 86:3796–3801. doi:10.3168/jds.S0022-0302(03)73987-3.

Broderick, G. A. 2003. Effects of varying dietary protein and energy levels on the production of lactating dairy cows. J. Dairy Sci. 86:1370–1381. doi:10.3168/jds.S0022-0302(03)73721-7.

Broderick, G. A., N. D. Luchini, S. M. Reynal, G. A. Varga, and V. A. Ishler. 2008. Effect on production of replacing dietary starch with sucrose in lactating dairy cows. J. Dairy Sci. 91:4801–4810. doi:10.3168/jds.2008-1480.

Colucci, P. E., L. E. Chase, and P. J. Van Soest. 1982. Feed intake, apparent diet digestibility, and rate of particulate passage in dairy cattle. J. Dairy Sci. 65:1445–1456. doi:10.3168/jds.S0022-0302(82)82367-9.

Devkota, S., Y. Wang, M. W. Musch, V. Leone, H. Fehlner-Peach, A. Nadimpalli, D. A. Antonopoulos, B. Jabri, and E. B. Chang. 2012. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in il10−/− mice. Nature. 487:104–108. doi:10.1038/nature11225.

Elliot, J. M., H. W. Symonds, and B. Pike. 1985. Effect on feed intake of infusing sodium propionate or sodium acetate into a mesenteric vein of cattle. J. Dairy Sci. 68:1165–1170. doi:10.3168/jds.S0022-0302(85)80943-7.

Farningham, D. A. H., and C. C. Whyte. 1993. The role of propionate and acetate in the control of food intake in sheep. Br. J. Nutr. 70:37–46. doi:10.1079/BJN19930103.

Haas, B. J., D. Gevers, A. M. Earl, M. Feldgarden, D. V. Ward, G. Giannoukos, D. Ciulla, D. Tabbaa, S. K. Highlander, E. Sodergren, B. Methe, T. Z. DeSantis, The Human Microbiome Consortium, J. F. Petrosino, R. Knight, and B. W. Birren. 2011. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res. 21:494–504. doi:10.1101/gr.112730.110.

Helbling, D. E., M. Ackermann, K. Fenner, H.-P. E. Kohler, and D. R. Johnson. 2012. The activity level of a microbial community function can be predicted from its metatranscriptome. ISME J. 6:902–904. doi:10.1038/ismej.2011.158.

Henderson, G., F. Cox, S. Ganesh, A. Jonker, W. Young, P. H. Janssen, and Global Rumen Census Collaborators. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Sci. Rep. 5:14567. doi:10.1038/srep14567.

Hildebrandt, M. A., C. Hoffmann, S. A. Sherrill–Mix, S. A. Keilbaugh, M. Hamady, Y. Chen, R. Knight, R. S. Ahima, F. Bushman, and G. D. Wu. 2009. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology. 137:1716-1724.e2. doi:10.1053/j.gastro.2009.08.042.

Hungate, R. E., M. P. Bryant, and R. A. Mah. 1964. The rumen bacteria and protozoa. Annu. Rev. Microbiol. 18:131–166. doi:10.1146/annurev.mi.18.100164.001023.

Hurvich, C. M., and C.-L. Tsai. 1989. Regression and time series model selection in small samples. Biometrika. 76:297–307. doi:10.1093/biomet/76.2.297.

Indugu, N., B. Vecchiarelli, L. D. Baker, J. D. Ferguson, J. K. Vanamala, and D. W. Pitta. 2017. Comparison of rumen bacterial communities in dairy herds of different production. BMC Microbiol. 17:190. doi:10.1186/s12866-017-1098-z.

Jami, E., and I. Mizrahi. 2012. Composition and similarity of bovine rumen microbiota across individual animals. P. López-García, editor. PLoS ONE. 7:e33306. doi:10.1371/journal.pone.0033306.

Jami, E., B. A. White, and I. Mizrahi. 2014. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. PloS One. 9:e85423. doi:10.1371/journal.pone.0085423.

Jewell, K. A., C. A. McCormick, C. L. Odt, P. J. Weimer, and G. Suen. 2015. Ruminal bacterial community composition in dairy cows is dynamic over the course of two lactations and correlates with feed efficiency. Appl. Environ. Microbiol. 81:4697–4710. doi:10.1128/AEM.00720-15.

Kalscheur, K. F., J. H. Vandersall, R. A. Erdman, R. A. Kohn, and E. Russek-Cohen. 1999. Effects of dietary crude protein concentration and degradability on milk production responses of early, mid, and late lactation dairy cows. J. Dairy Sci. 82:545–554. doi:10.3168/jds.S0022-0302(99)75266-5.

Klevenhusen, F., R. M. Petri, M.-T. Kleefisch, R. Khiaosa-ard, B. U. Metzler-Zebeli, and Q. Zebeli. 2017. Changes in fibre-adherent and fluid-associated microbial communities and fermentation profiles in the rumen of cattle fed diets differing in hay quality and concentrate amount. FEMS Microbiol. Ecol. 93. doi:10.1093/femsec/fix100. Available from: http://academic.oup.com/femsec/article/doi/10.1093/femsec/fix100/4067812/Changes-in-fibreadherent-and-fluidassociated

Krause, D. O., T. G. Nagaraja, A. D. G. Wright, and T. R. Callaway. 2013. Board-invited review: Rumen microbiology: Leading the way in microbial ecology. J. Anim. Sci. 91:331–341. doi:10.2527/jas.2012-5567.

Krehbiel, C. R., R. A. McCoy, R. A. Stock, T. J. Klopfenstein, D. H. Shain, and R. P. Huffman. 1995. Influence of grain type, tallow level, and tallow feeding system on feedlot cattle performance. J. Anim. Sci. 73:2916–2921. doi:10.2527/1995.73102916x.

de La Serre, C. B., C. L. Ellis, J. Lee, A. L. Hartman, J. C. Rutledge, and H. E. Raybould. 2010. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. Am. J. Physiol.-Gastrointest. Liver Physiol. 299:G440–G448. doi:10.1152/ajpgi.00098.2010.

Law, R. A., F. J. Young, D. C. Patterson, D. J. Kilpatrick, A. R. G. Wylie, and C. S. Mayne. 2009. Effect of dietary protein content on animal production and blood metabolites of dairy cows during lactation. J. Dairy Sci. 92:1001–1012. doi:10.3168/jds.2008-1155.

Li, F., and L. L. Guan. 2017. Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. A. J. McBain, editor. Appl. Environ. Microbiol. 83:e00061-17. doi:10.1128/AEM.00061-17.

Lin, L. I.-K. 1989. A concordance correlation coefficient to evaluate reproducibility. Biometrics. 45:255–268. doi:10.2307/2532051.

Mao, S. Y., R. Y. Zhang, D. S. Wang, and W. Y. Zhu. 2013. Impact of subacute ruminal acidosis (SARA) adaptation on rumen microbiota in dairy cattle using pyrosequencing. Anaerobe. 24:12–19. doi:10.1016/j.anaerobe.2013.08.003.

McAllister, T. A., R. C. Phillippe, L. M. Rode, and K.-J. Cheng. 1993. Effect of the protein matrix on the digestion of cereal grains by ruminal microorganisms. J. Anim. Sci. 71:205–212. doi:10.2527/1993.711205x.

Mullins, C. R., L. K. Mamedova, A. J. Carpenter, Y. Ying, M. S. Allen, I. Yoon, and B. J. Bradford. 2013. Analysis of rumen microbial populations in lactating dairy cattle fed diets varying in carbohydrate profiles and Saccharomyces cerevisiae fermentation product. J. Dairy Sci. 96:5872–5881. doi:10.3168/jds.2013-6775.

Myer, P. R., T. P. Smith, J. E. Wells, L. A. Kuehn, and H. C. Freetly. 2015. Rumen microbiome from steers differing in feed efficiency. PloS One. 10:e0129174. doi:doi.org/10.1371/journal.pone.0129174.

Patra, A. K. 2013. The effect of dietary fats on methane emissions, and its other effects on digestibility, rumen fermentation and lactation performance in cattle: A meta-analysis. Livest. Sci. 155:244–254. doi:10.1016/j.livsci.2013.05.023.

Penner, G. B., and M. Oba. 2009. Increasing dietary sugar concentration may improve dry matter intake, ruminal fermentation, and productivity of dairy cows in the postpartum phase of the transition period. J. Dairy Sci. 92:3341–3353. doi:10.3168/jds.2008-1977.

Pitta, D. W., N. Indugu, S. Kumar, B. Vecchiarelli, R. Sinha, L. D. Baker, B. Bhukya, and J. D. Ferguson. 2016. Metagenomic assessment of the functional potential of the rumen microbiome in Holstein dairy cows. Anaerobe. 38:50–60. doi:10.1016/j.anaerobe.2015.12.003.

R Core Team. 2017. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Roman-Garcia, Y., R. R. White, and J. L. Firkins. 2016. Meta-analysis of postruminal microbial nitrogen flows in dairy cattle. I. Derivation of equations. J. Dairy Sci. 99:7918–7931. doi:10.3168/jds.2015-10661.

Shabat, S. K. B., G. Sasson, A. Doron-Faigenboim, T. Durman, S. Yaacoby, M. E. Berg Miller, B. A. White, N. Shterzer, and I. Mizrahi. 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME J. 10:2958–2972. doi:10.1038/ismej.2016.62.

Sheperd, A. C., and D. K. Combs. 1998. Long-term effects of acetate and propionate on voluntary feed intake by midlactation cows. J. Dairy Sci. 81:2240–2250. doi:10.3168/jds.S0022-0302(98)75803-5.

Tremblay, J., K. Singh, A. Fern, E. S. Kirton, S. He, T. Woyke, J. Lee, F. Chen, J. L. Dangl, and S. G. Tringe. 2015. Primer and platform effects on 16S rRNA tag sequencing. Front. Microbiol. 6:771. doi:10.3389/fmicb.2015.00771.

Turnbaugh, P. J., M. Hamady, T. Yatsunenko, B. L. Cantarel, A. Duncan, R. E. Ley, M. L. Sogin, W. J. Jones, B. A. Roe, J. P. Affourtit, M. Egholm, B. Henrissat, A. C. Heath, R. Knight, and J. I. Gordon. 2009. A core gut microbiome in obese and lean twins. Nature. 457:480–484. doi:10.1038/nature07540.

Turnbaugh, P. J., R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 444:1027–1031. doi:10.1038/nature05414.

Van Horn, H. H., C. A. Zometa, C. J. Wilcox, S. P. Marshall, and B. Harris. 1979. Complete rations for dairy cattle. VIII. Effect of percent and source of protein on milk yield and ration digestibility. J. Dairy Sci. 62:1086–1093. doi:10.3168/jds.S0022-0302(79)83379-2.

Weimer, P. J., D. M. Stevenson, H. C. Mantovani, and S. L. C. Man. 2010. Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents. J. Dairy Sci. 93:5902–5912. doi:10.3168/jds.2010-3500.

White, R. R., M. B. Hall, J. L. Firkins, and P. J. Kononoff. 2017. Physically adjusted neutral detergent fiber system for lactating dairy cow rations. II: Development of feeding recommendations. J. Dairy Sci. 100:9569–9584. doi:10.3168/jds.2017-12766.

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. doi:10.3168/jds.2015-10662.

Whiting, F. 1957. The effect of concentrate to hay ratio and other ration factors on the feedlot performance of beef cattle. Can. J. Anim. Sci. 37:50–57. doi:10.4141/cjas57-007.

Zhang, J., H. Shi, Y. Wang, S. Li, Z. Cao, S. Ji, Y. He, and H. Zhang. 2017. Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in Holstein heifers. Front. Microbiol. 8:2206. doi:10.3389/fmicb.2017.02206.

TABLES

Table 1. Summary of data yielded by literature search that was utilized in the meta-analysis

Item	Variable	*n*	Mean	SD	Minimum	Maximum
Animal characteristics	DMI, kg/d	89	17.8	7.0	5.8	30.2
	MY, kg/d	61	33.0	9.3	16.4	70.7
	ADG, kg/d	22	1.0	0.60	0.06	2.1
Diet characteristics[1]	DM	102	64.5	22.5	17.7	94.2
	CP	136	15.8	3.19	5.32	24.1
	NDF	122	38.1	11.9	21.8	68.8
	ADF	92	25.4	8.94	10.3	45.4
	NFC	95	37.4	7.93	14.3	58.3
	Starch	95	21.9	10.3	0.0	59.1
	Ash	106	6.87	1.94	2.53	11.0
	Forage	177	61.2	25.1	0.0	100.0
Microbial characteristics[2]	Bacteroidetes	139	44.7	21.7	0.0	98.0
	Firmicutes	139	38.7	20.6	2.04	86.9
	Proteobacteria	113	3.35	4.35	0.0	28.6
	Chao1 richness	87	6995	17,184	181	92,297
	Shannon diversity	95	5.79	2.52	0.67	11.9

[1]All expressed as a percentage on a DM basis except DM, which is expressed on an AF basis.
[2]Bacteroidetes, Firmicutes, and Proteobacteria are expressed as % relative abundance.

Table 2. Parameter estimates in models of MY using diet, microbial, and experimental variable sets individually and in combination

Item[1]	Diet	Microbial	Microbial + DMI	Experimental	Diet + Microbial	Diet + Experimental[2]	Microbial + Experimental[3,4]	Diet + Microbial + Experimental
Model no.	1	2	3	4	5	6	7	8
Intercept	−1.80 (0.76)	11.8 (0.17)	11.3 (0.023)	−947.9 (0.57)	−10.3 (0.22)	−804.1 (0.59)	−5.62 (0.74)	102.8 (0.0088)
DMI, kg/d	0.480 (0.0066)		0.614 (0.001)		0.659 (0.026)	0.550 (0.0024)		0.283 (0.051)
CP, % DM	1.48 (<0.001)				1.76 (0.0016)	1.25 (0.0083)		1.77 (<0.001)
NFC, % DM						0.115 (0.058)		
Bacteroidetes		−0.280 (0.054)	0.124 (0.008)					
Firmicutes		0.73 (0.0041)	0.0924 (0.089)				0.772 (0.022)	−1.26 (0.023)
B:F		8.90 (0.0045)					8.04 (0.0075)	−37.4 (0.0024)
Proteobacteria		−1.26 (0.020)			0.222 (0.22)			
Chao1 richness		−0.00132 (0.0048)					−1.25 (0.0091)	
Year				0.488 (0.55)		0.394 (0.59)		
Fluid fraction							53.2 (0.081)	−96.5 (0.012)
Solid fraction							50.3 (0.62)	−107.2 (0.0069)
Firmicutes × Fluid fraction							−0.952 (0.054)	1.10 (0.044)
Firmicutes × Solid fraction							−0.992 (0.59)	1.25 (0.027)
B:F × Fluid fraction							−12.4 (0.075)	35.6 (0.0036)
B:F × Solid fraction							−8.40 (0.73)	39.3 (0.0025)
Fit statistics								
n	38	31	36	61	27	36	38	34
CCC	0.98	0.98	0.98	0.80	0.99	0.99	0.98	0.99
uCCC	0.54	0.49	0.58	0.49	0.49	0.54	0.30	0.71
AICc	168.24	177.04	177.88	391.09	126.28	168.80	190.24	150.57
$\hat{\sigma}_s$	5.20	5.71	5.10	7.18	5.78	5.55	7.12	5.72
$\hat{\sigma}_e$	0.92	1.58	1.07	3.56	0.95	0.80	1.60	0.64
$\hat{\sigma}_s/\hat{\sigma}_e$	5.68	3.60	4.75	2.02	6.09	6.96	4.46	8.98

P values are given in parentheses.

[1]B:F = Bacteroidetes-Firmicutes ratio; CCC = concordance correlation coefficient; uCCC = uncalibrated CCC; AICc = corrected Akaike information criterion; $\hat{\sigma}_s$ = standard deviation for study; $\hat{\sigma}_e$ = standard deviation for error.

[2]Significant slope-by-study interactions for CP, NFC, and year ($P < 0.05$).

[3]Significant slope-by-study interaction for B:F ($P < 0.05$).

[4]Chao1 variable divided by 1,000 to correct a scaling issue.

Table 3. Parameter estimates in models of dairy feed efficiency (FE) using diet, microbial, and experimental variable sets individually and in combination

Item[1]	Diet[2]	Microbial	Experimental	Diet + Microbial	Diet + Experimental	Microbial + Experimental
Model no.	1	2	3	4	5	6
Intercept	2.23 (<0.001)	1.90 (<0.001)	18.7 (0.69)	2.08 (<0.001)	−127.0 (0.076)	5.37 (0.002)
DM, %	0.00388 (<0.001)				0.00387 (<0.001)	
CP, % DM	−0.0309 (<0.001)				−0.0315 (<0.001)	
NDF, % DM	−0.0211 (<0.001)				−0.0211 (<0.001)	
ADF, % DM	−0.00706 (0.0012)				−0.0721 (<0.001)	
Starch, % DM	0.0185 (<0.001)			−0.0037 (0.47)	0.0185 (<0.001)	
EE, % DM	−0.0176 (<0.001)				−0.0177 (<0.001)	
Shannon diversity		−0.0552 (0.031)		−0.0712 (0.045)		−0.441 (0.019)
Year			−0.00854 (0.72)		0.0640 (0.073)	
Fluid fraction						−3.424 (0.034)
Solid fraction						−3.528 (0.033)
Shannon diversity × Fluid fraction						0.361 (0.052)
Shannon diversity × Solid fraction						0.377 (0.052)
Fit statistics						
n	12	25	40	25	12	25
CCC	>0.99	0.93	0.93	0.94	>0.99	0.94
uCCC	0.65	0.26	0.012	0.24	0.88	0.69
AICc	101.15	−28.28	−42.18	−16.72	234.74	−9.31
$\hat{\sigma}_s$	0.12	0.17	0.18	0.20	0.065	0.11
$\hat{\sigma}_e$	0.0013	0.05	0.06	0.051	0.0013	0.05
$\hat{\sigma}_s/\hat{\sigma}_e$	82.25	3.28	2.83	3.94	50.1	2.13

P values are given in parentheses. Model of Diet + Microbial + Experimental variables could not be derived due to high values for variance inflation factor (VIF).

[1]CCC = concordance correlation coefficient; uCCC = uncalibrated CCC; AICc = corrected Akaike information criterion; $\hat{\sigma}_s$ = standard deviation for study; $\hat{\sigma}_e$ = standard deviation for error.

[2]Significant slope-by-study interaction for CP ($P < 0.05$).

Table 4. Parameter estimates in models of ADG using diet, microbial, and experimental variable sets individually and in combination

Item[1]	Diet	Microbial	Experimental	Diet + Microbial[2]	Diet + Experimental	Microbial + Experimental	Diet + Microbial + Experimental[2]
Model no.	1	2	4	5	6	7	8
Intercept	−0.873 (0.12)	1.81 (0.010)	1.37 (0.014)	2.05 (<0.001)	−0.0363 (0.83)	1.93 (<0.001)	2.10 (<0.001)
DMI, kg/d	0.0909 (<0.001)						
NFC, % DM	0.0272 (<0.001)				0.0330 (<0.001)		
Forage, % DM				−0.010 (0.0054)			−0.0102 (0.0097)
B:F		−0.265 (0.028)		−0.175 (0.068)		−0.168 (0.087)	−0.192 (0.088)
B:F × Solid fraction							−0.139 (0.67)
Bos indicus breed type			−0.909 (0.23)		0.136 (0.41)	−0.928 (0.0062)	
Fit statistics							
n	10	10	22	10	14	10	10
CCC	1.0	0.93	0.90	0.92	0.91	0.92	0.92
uCCC	0.92	0.88	0.74	0.92	0.91	0.92	0.92
AICc	−5.86	27.85	30.11	42.04	28.75	33.24	57.39
$\hat{\sigma}_s$	0.29	0.46	0.52	<0.001	<0.001	<0.001	<0.001
$\hat{\sigma}_e$	0.03	1.18	1.15	1.17	0.930	1.19	1.24
$\hat{\sigma}_s/\hat{\sigma}_e$	8.96	0.39	0.45	<0.001	<0.001	<0.001	<0.001

P values are given in parentheses. Model with microbial + DMI variables possessed an insufficient number of observations for derivation. Intercepts for Models 4, 6, and 7 are represented by the estimate for beef breed type. [1]B:F = Bacteroidetes-Firmicutes ratio; CCC = concordance correlation coefficient; uCCC = uncalibrated CCC; AICc = corrected Akaike information criterion; $\hat{\sigma}_s$ = standard deviation for study; $\hat{\sigma}_e$ = standard deviation for error.

[2]Significant slope-by-study interaction for forage ($P < 0.05$).

Table 5. Parameter estimates in models of beef feed efficiency (FE) using diet, microbial, and experimental variable sets individually and in combination

Item[1]	Diet	Microbial
Model no.	1	2
Intercept	0.0313 (<0.001)	0.173 (0.014)
Starch, % DM	0.00222 (<0.001)	
EE, % DM	0.000968 (0.053)	
B:F		−0.00425 (0.77)
Fit statistics		
n	10	6
CCC	>0.99	0.044
uCCC	>0.99	0.044
AICc	−29.29	35.31
$\hat{\sigma}_s$	<0.001	<0.001
$\hat{\sigma}_e$	0.00689	0.126
$\hat{\sigma}_s/\hat{\sigma}_e$	<0.001	<0.001

P values are given in parentheses. Models with experimental variables, diet + microbial variables, diet + experimental variables, microbial + experimental variables, and diet + microbial + experimental variables possessed insufficient numbers of observations for derivation.

[1]B:F = Bacteroidetes-Firmicutes ratio; CCC = concordance correlation coefficient; uCCC = uncalibrated CCC; AICc = corrected Akaike information criterion; $\hat{\sigma}_s$ = standard deviation for study; $\hat{\sigma}_e$ = standard deviation for error.

Table 6. Parameter estimates in models of DMI using diet, microbial, and experimental variable sets individually and in combination

Item[1]	Diet[2]	Microbial	Experimental	Diet + Microbial	Diet + Experimental[4]	Microbial + Experimental	Diet + Microbial + Experimental
Model no.	1	2	3	4	5	6	7
Intercept	−24.2 (0.31)	19.1 (<0.001)	−2180.5 (0.024)	15.4 (<0.001)	−24.3 (0.30)	−2105.08 (0.011)	10.4 (0.0068)
DM, %							−0.0296 (0.22)
Starch, % DM	−0.378 (1.11e-6)			−0.296 (0.0026)	−0.355 (<0.001)		
NFC, % DM	0.25 (0.0060)			0.285 (0.0050)	0.217 (0.018)		
OM, % DM	0.480 (0.059)				0.432 (0.083)		
Forage, % DM	−0.0590 (0.014)				−0.0674 (0.0050)		
B:F				−0.191 (0.080)			
Shannon diversity		−0.678 (0.099)				0.465 (0.45)	1.37 (0.022)
Year			1.09 (0.023)			1.05 (0.011)	
Fluid fraction						5.84 (0.17)	8.51 (0.045)
Solid fraction						5.96 (0.19)	8.80 (0.042)
Bos indicus breed type						−8.06 (0.060)	−6.43 (0.11)
Dairy breed type			10.1 (<0.001)		8.31 (0.018)	7.16 (0.01)	5.36 (0.071)
Shannon diversity × Fluid fraction						−1.86 (0.015)	−2.36 (0.0015)
Shannon diversity × Solid fraction						−1.89 (0.016)	−2.40 (0.0013)
Fit statistics							
n	74	51	89	52	74	51	45
CCC	0.99	0.99	0.98	0.99	0.99	0.99	0.99
uCCC	0.69	0.46	0.78	0.66	0.76	0.93	0.90
AICc	349.47	225.40	423.82	230.28	335.33	203.85	165.49
$\widehat{\sigma}_s$	6.79	7.24	5.38	5.41	5.41	2.92	2.66
$\widehat{\sigma}_e$	1.21	1.07	1.64	1.02	1.21	1.05	0.77
$\widehat{\sigma}_s/\widehat{\sigma}_e$	5.63	6.74	3.29	5.33	4.48	2.77	3.44

P values are given in parentheses. Intercepts for Models 3, 5, 6, and 7 are represented by the estimate for beef breed type.

[1]CCC = concordance correlation coefficient; uCCC = uncalibrated CCC; AICc = corrected Akaike information criterion; $\widehat{\sigma}_s$ = standard deviation for study; $\widehat{\sigma}_e$ = standard deviation for error.

[2]Significant slope-by-study interaction for forage ($P < 0.05$).

Table 7. Parameter estimates in models of Bacteroidetes relative abundance using diet and experimental variable sets individually and in combination

Item[1]	Diet	Experimental	Diet + Experimental
Model no.	1	2	3
Intercept	31.9 (<0.001)	−5115.5 (0.071)	−5626.3 (0.097)
CP, % DM	0.648 (0.19)		
NDF, % DM			−0.191 (0.15)
Year		2.56 (0.069)	2.82 (0.094)
Bos indicus breed type		26.6 (0.030)	36.2 (0.012)
Dairy breed type		2.13 (0.75)	0.69 (0.92)
Fit statistics			
n	124	139	112
CCC	0.94	0.94	0.95
uCCC	0.60	0.65	0.71
AICc	981.78	1087.53	871.54
$\hat{\sigma}_s$	21.33	19.21	19.38
$\hat{\sigma}_e$	8.44	8.53	8.15
$\hat{\sigma}_s/\hat{\sigma}_e$	2.52	2.25	2.37

P-values are given in parentheses. Intercepts for Models 2 and 3 are represented by the estimate for beef breed type.

[1]CCC = concordance correlation coefficient; uCCC = uncalibrated CCC; AICc = corrected Akaike information criterion; $\hat{\sigma}_s$ = standard deviation for study; $\hat{\sigma}_e$ = standard deviation for error.

Table 8. Parameter estimates in models of Firmicutes relative abundance using diet and experimental variable sets individually and in combination

Item[1]	Diet	Experimental	Diet + Experimental
Model no.	1	2	3
Intercept	48.8 (<0.001)	5884.1 (0.013)	6091.4 (0.033)
DM, %	−0.0948 (0.24)		−0.082 (0.30)
Year		−2.90 (0.014)	−3.00 (0.034)
Bos indicus breed type		−29.5 (0.0052)	−30.5 (0.0065)
Dairy breed type		−3.15 (0.61)	−4.19 (0.55)
Fit statistics			
n	124	139	124
CCC	0.88	0.89	0.88
uCCC	0.63	0.72	0.75
AICc	1014.40	1111.28	993.11
$\hat{\sigma}_s$	18.67	15.10	15.34
$\hat{\sigma}_e$	10.28	10.28	10.30
$\hat{\sigma}_s/\hat{\sigma}_e$	1.82	1.47	1.49

P values are given in parentheses. Intercepts for Models 2 and 3 are represented by the estimate for beef breed type.

[1]CCC = concordance correlation coefficient; uCCC = uncalibrated CCC; AICc = corrected Akaike information criterion; $\hat{\sigma}_s$ = standard deviation for study; $\hat{\sigma}_e$ = standard deviation for error.

Table 9. Parameter estimates in models of the Bacteroidetes–Firmicutes ratio (B:F) using diet and experimental variable sets individually and in combination

Item[1]	Diet	Experimental[2]	Diet + Experimental[2]
Model no.	1	2	3
Intercept	2.12 (0.18)	1.85 (0.21)	1.12 (0.54)
DM, %	0.0115 (0.253)		0.0114 (0.26)
Bos indicus breed type		12.4 (0.0012)	12.3 (0.0032)
Dairy breed type		−0.673 (0.58)	−0.70 (0.60)
Fit statistics			
n	124	139	124
CCC	0.98	0.98	0.98
uCCC	0.66	0.61	0.61
AICc	565.53	603.0	552.28
$\hat{\sigma}_s$	8.06	2.43	6.98
$\hat{\sigma}_e$	1.17	1.12	1.17
$\hat{\sigma}_s/\hat{\sigma}_e$	6.89	5.73	5.96

P values are given in parentheses. Intercepts for Models 2 and 3 are represented by the estimate for beef breed type.

[1]CCC = concordance correlation coefficient; uCCC = uncalibrated CCC; AICc = corrected Akaike information criterion; $\hat{\sigma}_s$ = standard deviation for study; $\hat{\sigma}_e$ = standard deviation for error.

[2]Significant slope-by-study interaction for *Bos indicus* breed type ($P < 0.05$).

Table 10. Parameter estimates in models of Proteobacteria relative abundance using diet and experimental variable sets individually and in combination

Item[1]	Diet	Experimental	Diet + Experimental
Model no.	1	2	3
Intercept	−8.46 (0.0067)	6.23 (<0.001)	8.75 (<0.001)
CP, % DM			−0.111 (0.42)
EE, % DM	3.05 (<0.001)		
Forage, % DM	0.0312 (0.023)		
Bos indicus breed type		−5.13 (0.060)	−6.27 (0.025)
Dairy breed type		−2.56 (0.12)	−3.60 (0.025
Fit statistics			
n	36	113	100
CCC	0.98	0.73	0.86
uCCC	0.69	0.67	0.68
AICc	180.60	620.43	485.04
$\hat{\sigma}_s$	5.27	3.40	3.58
$\hat{\sigma}_e$	1.28	3.03	1.96
$\hat{\sigma}_s/\hat{\sigma}_e$	4.13	1.12	1.83

P values are given in parentheses. Intercepts for Models 2 and 3 are represented by the estimate for beef breed type.

[1]CCC = concordance correlation coefficient; uCCC = uncalibrated CCC; AICc = corrected Akaike information criterion; $\hat{\sigma}_s$ = standard deviation for study; $\hat{\sigma}_e$ = standard deviation for error.

Table 11. Parameter estimates in models of Chao1 richness using diet and experimental variable sets individually and in combination

Item[1]	Diet[2]	Experimental[3]	Diet + Experimental[2]
Model no.	1	2	3
Intercept	152282.2 (<0.001)	7010.4 (0.082)	147444.03 (<0.001)
CP, % DM	−204.9 (0.072)		−207.99 (0.070)
Starch, % DM	75.1 (0.069)		74.16 (0.075)
OM, % DM	−1551.5 (<0.001)		−1538.9 (<0.001)
Bos indicus breed type		−6627.2 (0.61)	−132.3 (0.99)
Dairy breed type		81.5 (0.97)	5353.5 (0.59)
Fit statistics			
n	77	88	77
CCC	0.998	1.0	1.0
uCCC	0.69	0.67	0.69
AICc	1416.58	1646.52	1379.46
$\hat{\sigma}_s$	17768.62	16962.06	18593.34
$\hat{\sigma}_e$	1400.89	1681.32	1401.0
$\hat{\sigma}_s/\hat{\sigma}_e$	12.68	10.09	13.27

P values are given in parentheses. Intercepts for Models 2 and 3 are represented by the estimate for beef breed type.

[1]CCC = concordance correlation coefficient; uCCC = uncalibrated CCC; AICc = corrected Akaike information criterion; $\hat{\sigma}_s$ = standard deviation for study; $\hat{\sigma}_e$ = standard deviation for error.

[2]Significant slope-by-study interactions for OM and starch ($P < 0.05$).

[3]Significant slope-by-study interaction for dairy breed type ($P < 0.05$).

Table 12. Parameter estimates in models of Shannon diversity using diet and experimental variable sets individually and in combination

Item[1]	Diet[2]	Experimental	Diet + Experimental[2]
Model no.	1	2	3
Intercept	4.29 (<0.001)	−911.8 (0.014)	4.23 (<0.001)
NDF, % DM	0.0316 (<0.001)		0.032 (<0.001)
Year		0.456 (0.014)	
Bos indicus breed type		−0.360 (0.84)	−0.510 (0.80)
Dairy breed type		0.0952 (0.85)	0.136 (0.78)
Fit statistics			
n	89	95	89
CCC	0.98	0.99	0.99
uCCC	0.63	0.58	0.63
AICc	232.62	250.38	233.50
$\hat{\sigma}_s$	2.55	2.32	2.59
$\hat{\sigma}_e$	0.42	0.47	0.42
$\hat{\sigma}_s/\hat{\sigma}_e$	6.07	4.97	6.11

P values are given in parentheses. Intercepts for Models 2 and 3 are represented by the estimate for beef breed type.

[1]CCC = concordance correlation coefficient; uCCC = uncalibrated CCC; AICc = corrected Akaike information criterion; $\hat{\sigma}_s$ = standard deviation for study; $\hat{\sigma}_e$ = standard deviation for error.

[2]Significant slope-by-study interaction for NDF ($P < 0.05$).

Supplementary Tables

Supplementary Table S1. Publications used in the dataset

No.	Citation
1	AlZahal, O., F. Li, L. L. Guan, N. D. Walker, and B. W. McBride. 2017. Factors influencing ruminal bacterial community diversity and composition and microbial fibrolytic enzyme abundance in lactating dairy cows with a focus on the role of active dry yeast. J. Dairy Sci. 100:4377–4393. doi:10.3168/jds.2016-11473
2	Azad, E., N. Narvaez, H. Derakhshani, A. Y. Allazeh, Y. Wang, T. A. McAllister, and E. Khafipour. 2017. Effect of *Propionibacterium acidipropionici* P169 on the rumen and faecal microbiota of beef cattle fed a maize-based finishing diet. Benef. Microbes. 8:785–799. doi:10.3920/BM2016.0145
3	Bainbridge, M. L., L. M. Cersosimo, A.-D. G. Wright, and J. Kraft. 2016. Rumen bacterial communities shift across a lactation in Holstein, Jersey and Holstein × Jersey dairy cows and correlate to rumen function, bacterial fatty acid composition and production parameters. FEMS microbiology. 92:fiw059. doi:10.1093/femsec/fiw059
4	Callaway, T. R., S. E. Dowd, T. S. Edrington, R. C. Anderson, N. Krueger, N. Bauer, P. J. Kononoff, and D. J. Nisbet. 2010. Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing1. J. Anim. Sci. 88:3977–3983. doi:10.2527/jas.2010-2900
5	Carberry, C. A., D. A. Kenny, S. Han, M. S. McCabe, and S. M. Waters. 2012. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. Appl. Environ. Microbiol. 78:4949–4958. doi:10.1128/AEM.07759-11
6	Castillo-Lopez, E., C. J. R. Jenkins, N. D. Aluthge, W. Tom, P. J. Kononoff, and S. C. Fernando. 2017. The effect of regular or reduced-fat distillers grains with solubles on rumen methanogenesis and the rumen bacterial community. Journal of applied microbiology. 123:1381–1395. doi:10.1111/jam.13583
7	Castillo-Lopez, E., J. Moats, N. D. Aluthge, H. A. Ramirez-Ramirez, D. A. Christensen, T. Mutsvangwa, and Penner G B Fernando S. 2017b. Effect of partially replacing a barley-based concentrate with flaxseed-based products on the rumen bacterial population of lactating Holstein dairy cows. Journal of applied microbiology. 124:42–57. doi:10.1111/jam.13630
8	Castillo-Lopez, E., H. A. Ramirez Ramirez, T. J. Klopfenstein, C. L. Anderson, N. D. Aluthge, S. C. Fernando, T. Jenkins, and P. J. Kononoff. 2014. Effect of feeding dried distillers grains with solubles on ruminal biohydrogenation, intestinal fatty acid profile, and gut microbial diversity evaluated through DNA pyro-sequencing. J. Anim. Sci. 92:733–743. doi:10.2527/jas.2013-7223
9	Fernando, S. C., H. T. Purvis 2nd, F. Z. Najar, L. O. Sukharnikov, C. R. Krehbiel, T. G. Nagaraja, B. A. Roe, and U. Desilva. 2010. Rumen microbial population dynamics during adaptation to a high-grain diet. Appl. Environ. Microbiol. 76:7482–7490. doi:10.1128/AEM.00388-10
10	Fouts, D. E., S. Szpakowski, J. Purushe, M. Torralba, R. C. Waterman, M. D. MacNeil, L. J. Alexander, and K. E. Nelson. 2012. Next generation sequencing to define prokaryotic and fungal diversity in the bovine rumen. PLoS One. 7:e48289. doi:10.1371/journal.pone.0048289
11	Golder, H. M., S. E. Denman, C. McSweeney, P. Celi, and I. J. Lean. 2014. Ruminal bacterial community shifts in grain-, sugar-, and histidine-challenged dairy heifers. J. Dairy Sci. 97:5131–5150. doi:10.3168/jds.2014-8003
12	Hernandez-Sanabria, E., L. A. Goonewardene, Z. Wang, O. N. Durunna, S. S. Moore, and L. L. Guan. 2012. Impact of feed efficiency and diet on adaptive variations in the bacterial community in the rumen fluid of cattle. Appl. Environ. Microbiol. 78:1203–1214. doi:10.1128/AEM.05114-11

Supplementary Table S1 cont'd.

No.	Citation
13	Hook, S. E., M. A. Steele, K. S. Northwood, J. Dijkstra, J. France, A.-D. G. Wright, and B. W. McBride. 2011. Impact of subacute ruminal acidosis (SARA) adaptation and recovery on the density and diversity of bacteria in the rumen of dairy cows. FEMS Microbiol. Ecol. 78:275–284. doi:10.1111/j.1574-6941.2011.01154.x
14	Indugu, N., B. Vecchiarelli, L. D. Baker, J. D. Ferguson, J. K. P. Vanamala, and D. W. Pitta. 2017. Comparison of rumen bacterial communities in dairy herds of different production. BMC Microbiol. 17:190. doi:10.1186/s12866-017-1098-z
15	Jami, E., B. A. White, and I. Mizrahi. 2014. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. PLoS One. 9:e85423. doi:10.1371/journal.pone.0085423
16	de Jesus, R. B. de, W. P. Omori, E. G. de M. Lemos, and J. A. M. de Souza. 2015. Bacterial diversity in bovine rumen by metagenomic 16S rDNA sequencing and scanning electron microscopy. Acta Sci., Anim. Sci. 37:251–257. doi:10.4025/actascianimsci.v37i3.26535
17	Jewell, K. A., C. A. McCormick, C. L. Odt, P. J. Weimer, and G. Suen. 2015. Ruminal Bacterial Community Composition in Dairy Cows Is Dynamic over the Course of Two Lactations and Correlates with Feed Efficiency. Appl. Environ. Microbiol. 81:4697–4710. doi:10.1128/AEM.00720-15
18	Jing, L., R. Zhang, Y. Liu, W. Zhu, and S. Mao. 2014. Intravenous lipopolysaccharide challenge alters ruminal bacterial microbiota and disrupts ruminal metabolism in dairy cattle. Br. J. Nutr. 112:170–182. doi:10.1017/S000711451400066X
19	Ji, S., H. Zhang, H. Yan, A. Azarfar, H. Shi, G. Alugongo, S. Li, Z. Cao, and Y. Wang. 2017. Comparison of rumen bacteria distribution in original rumen digesta, rumen liquid and solid fractions in lactating Holstein cows. J. Anim. Sci. Biotechnol. 8:16. doi:10.1186/s40104-017-0142-z
20	Khafipour, E., S. Li, J. C. Plaizier, and D. O. Krause. 2009. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. Appl. Environ. Microbiol. 75:7115–7124. doi:10.1128/AEM.00739-09
21	Kim, M., M. Morrison, and Z. Yu. 2011. Phylogenetic diversity of bacterial communities in bovine rumen as affected by diets and microenvironments. Folia Microbiol. 56:453–458. doi:10.1007/s12223-011-0066-5
22	Klevenhusen, F., R. M. Petri, M. T. Kleefisch, K. Ratchaneewan, B. U. Metzler-Zebeli, and Q. Zebeli. 2017. Changes in fibre-adherent and fluid-associated microbial communities and fermentation profiles in the rumen of cattle fed diets differing in hay quality and concentrate amount. FEMS microbiology. 93:fix100. doi:10.1093/femsec/fix100
23	Lee, H. J., J. Y. Jung, Y. K. Oh, S.-S. Lee, E. L. Madsen, and C. O. Jeon. 2012. Comparative survey of rumen microbial communities and metabolites across one caprine and three bovine groups, using bar-coded pyrosequencing and 1H nuclear magnetic resonance spectroscopy. Appl. Environ. Microbiol. 78:5983–5993. doi:10.1128/AEM.00104-12
24	Lima, F. S., G. Oikonomou, S. F. Lima, M. L. S. Bicalho, E. K. Ganda, J. C. de O. Filho, G. Lorenzo, P. Trojacanec, and R. C. Bicalhoa. 2015. Prepartum and postpartum rumen fluid microbiomes: characterization and correlation with production traits in dairy cows. Appl. Environ. Microbiol. 81:1327–1337. doi:10.1128/AEM.03138-14. doi:10.1128/AEM.03138-14
25	Li, R. W., S. Wu, R. L. Baldwin 6th, W. Li, and C. Li. 2012. Perturbation dynamics of the rumen microbiota in response to exogenous butyrate. PLoS One. 7:e29392. doi:10.1371/journal.pone.0029392

Supplementary Table S1 cont'd.

No.	Citation
26	Li, Y., S. Ma, X. Zhang, S. Huang, H. Yang, F. Zhao, W. Yi, X. Yang, S. Xin, X. Yi, B. Yang, Y. Tu, Y. Tian, and X. Lu. 2013. Evaluation of bacterial and archaeal diversity in the rumen of Xiangxi yellow cattle (Bos taurus) fed Miscanthus sinensis or common mixed feedstuff. Ann. Microbiol. 64:1385–1394. doi:10.1007/s13213-013-0783-x
27	Mao, S., M. Zhang, J. Liu, and W. Zhu. 2015. Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. Sci. Rep. 5:16116. doi:10.1038/srep16116
28	Mao, S. Y., R. Y. Zhang, D. S. Wang, and W. Y. Zhu. 2013. Impact of subacute ruminal acidosis (SARA) adaptation on rumen microbiota in dairy cattle using pyrosequencing. Anaerobe. 24:12–19. doi:10.1016/j.anaerobe.2013.08.003
29	McCann, J. C., L. M. Wiley, T. D. Forbes, F. M. Rouquette Jr, and L. O. Tedeschi. 2014. Relationship between the rumen microbiome and residual feed intake-efficiency of Brahman bulls stocked on bermudagrass pastures. PLoS One. 9:e91864. doi:10.1371/journal.pone.0091864
30	de Menezes, A. B., E. Lewis, M. O'Donovan, B. F. O'Neill, N. Clipson, and E. M. Doyle. 2011. Microbiome analysis of dairy cows fed pasture or total mixed ration diets. FEMS Microbiol. Ecol. 78:256–265. doi:10.1111/j.1574-6941.2011.01151.x
31	Mullins, C. R., L. K. Mamedova, A. J. Carpenter, Y. Ying, M. S. Allen, I. Yoon, and B. J. Bradford. 2013. Analysis of rumen microbial populations in lactating dairy cattle fed diets varying in carbohydrate profiles and Saccharomyces cerevisiae fermentation product. J. Dairy Sci. 96:5872–5881. doi:10.3168/jds.2013-6775
32	Myer, P. R., T. P. L. Smith, J. E. Wells, L. A. Kuehn, and H. C. Freetly. 2015. Rumen microbiome from steers differing in feed efficiency. PLoS One. 10:e0129174. doi:10.1371/journal.pone.0129174
33	de Oliveira, M. N. V., K. A. Jewell, F. S. Freitas, L. A. Benjamin, M. R. Tótola, A. C. Borges, C. A. Moraes, and G. Suen. 2013. Characterizing the microbiota across the gastrointestinal tract of a Brazilian Nelore steer. Vet. Microbiol. 164:307–314. doi:10.1016/j.vetmic.2013.02.013
34	Palmonari, A., D. M. Stevenson, D. R. Mertens, C. W. Cruywagen, and P. J. Weimer. 2010. pH dynamics and bacterial community composition in the rumen of lactating dairy cows. J. Dairy Sci. 93:279–287. doi:10.3168/jds.2009-2207
35	Patel, V., A. K. Patel, N. R. Parmar, A. B. Patel, B. Reddy, and C. G. Joshi. 2014. Characterization of the rumen microbiome of Indian Kankrej cattle (Bos indicus) adapted to different forage diet. Appl. Microbiol. Biotechnol. 98:9749–9761. doi:10.1007/s00253-014-6153-1
36	Petri, R. M., C. Mapiye, M. E. R. Dugan, and T. A. McAllister. 2014. Subcutaneous adipose fatty acid profiles and related rumen bacterial populations of steers fed red clover or grass hay diets containing flax or sunflower-seed. PLoS One. 9:e104167. doi:10.1371/journal.pone.0104167
37	Petri, R. M., T. Schwaiger, G. B. Penner, K. A. Beauchemin, R. J. Forster, J. J. McKinnon, and T. A. McAllister. 2013. Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. PLoS One. 8:e83424. doi:10.1371/journal.pone.0083424
38	Petri, R. M., R. J. Forster, W. Yang, J. J. McKinnon, and T. A. McAllister. 2012. Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage. J. Appl. Microbiol. 112:1152–1162. doi:10.1111/j.1365-2672.2012.05295.x
39	Pinloche, E., N. McEwan, J.-P. Marden, C. Bayourthe, E. Auclair, and C. J. Newbold. 2013. The effects of a probiotic yeast on the bacterial diversity and population structure in the rumen of cattle. PLoS One. 8:e67824. doi:10.1371/journal.pone.0067824

Supplementary Table S1 cont'd.

No.	Citation
40	Pitta, D. W., E. Pinchak, S. E. Dowd, J. Osterstock, V. Gontcharova, E. Youn, K. Dorton, I. Yoon, B. R. Min, J. D. Fulford, T. A. Wickersham, and D. P. Malinowski. 2009. Rumen bacterial diversity dynamics associated with changing from bermudagrass hay to grazed winter wheat diets. Microb. Ecol. 59:511–522. doi:10.1007/s00248-009-9609-6
41	Ramirez-Ramirez, H. A., E. Castillo Lopez, C. J. R. Jenkins, N. D. Aluthge, C. Anderson, S. C. Fernando, K. J. Harvatine, and P. J. Kononoff. 2016. Reduced-fat dried distillers grains with solubles reduces the risk for milk fat depression and supports milk production and ruminal fermentation in dairy cows. J. Dairy Sci. 99:1912–1928. doi:10.3168/jds.2015-9712
42	Ramirez-Ramirez, H. A., K. Nestor, L. O. Tedeschi, T. R. Callaway, S. E. Dowd, S. C. Fernando, and P. J. Kononoff. 2012. The effect of brown midrib corn silage and dried distillers' grains with solubles on milk production, nitrogen utilization and microbial community structure in dairy cows. Can. J. Anim. Sci. 92:365–380. doi:10.1139/CJAS2011-133
43	Romero-Pérez, G. A., K. H. Ominski, T. A. McAllister, and D. O. Krause. 2011. Effect of environmental factors and influence of rumen and hindgut biogeography on bacterial communities in steers. Appl. Environ. Microbiol. 77:258–268. doi:10.1128/AEM.01289-09
44	Sandri, M., C. Manfrin, A. Pallavicini, and B. Stefanon. 2014. Microbial biodiversity of the liquid fraction of rumen content from lactating cows. Animal. 8:572–579. doi:10.1017/S1751731114000056
45	Shabat, S. K. B., G. Sasson, A. Doron-Faigenboim, T. Durman, S. Yaacoby, M. E. Berg Miller, B. A. White, N. Shterzer, and I. Mizrahi. 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME J. 10:2958–2972. doi:10.1038/ismej.2016.62
46	Tajima, K., I. Nonaka, K. Higuchi, N. Takusari, M. Kurihara, A. Takenaka, M. Mitsumori, H. Kajikawa, and R. I. Aminov. 2007. Influence of high temperature and humidity on rumen bacterial diversity in Holstein heifers. Anaerobe. 13:57–64. doi:10.1016/j.anaerobe.2006.12.001
47	Thoetkiattikul, H., W. Mhuantong, T. Laothanachareon, S. Tangphatsornruang, V. Pattarajinda, L. Eurwilaichitr, and V. Champreda. 2013. Comparative analysis of microbial profiles in cow rumen fed with different dietary fiber by tagged 16S rRNA gene pyrosequencing. Curr. Microbiol. 67:130–137. doi:10.1007/s00284-013-0336-3
48	Wang, X., X. Li, C. Zhao, P. Hu, H. Chen, Z. Liu, G. Liu, and Z. Wang. 2012. Correlation between composition of the bacterial community and concentration of volatile fatty acids in the rumen during the transition period and ketosis in dairy cows. Appl. Environ. Microbiol. 78:2386–2392. doi:10.1128/AEM.07545-11
49	Wu, S., R. L. Baldwin, W. Li, C. Li, E. E. Connor, and R. W. Li. 2012. The bacterial community composition of the bovine rumen detected using pyrosequencing of 16S rRNA genes. Metagenomics. 1:1–11. doi:10.4303/mg/235571
50	Zened, A., S. Combes, L. Cauquil, J. Mariette, C. Klopp, O. Bouchez, A. Troegeler-Meynadier, and F. Enjalbert. 2013. Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. FEMS Microbiol. Ecol. 83:504–514. doi:10.1111/1574-6941.12011
51	Zhang, R., W. Zhu, W. Zhu, J. Liu, and S. Mao. 2013. Effect of dietary forage sources on rumen microbiota, rumen fermentation and biogenic amines in dairy cows. J. Sci. Food Agric. 94:1886–1895. doi:10.1002/jsfa.6508

Supplementary Table S2. Summary of explanatory variables used in predictions of animal performance[1]

Item	MY	Dairy FE	ADG	Beef FE	DMI
Diet variables					
DMI, kg/d	X		X		
DM, %		X			
OM, % DM					x
CP, % DM	X	X			
NDF, % DM		X			
ADF, % DM		X			
NFC, % DM			X		X
Starch, % DM		X		X	X
EE, % DM		X		X	
Forage, % DM					X
Microbial variables					
Bacteroidetes (% relative abundance)	X				
Firmicutes (% relative abundance)	X				
B:F[2]	X		X		x
Proteobacteria (% relative abundance)	X				
Chao1 richness	X				
Shannon diversity		X			x
Experimental variables					
Publication year		X			X
Sample type	X	x			X
Breed type			X		X

[1]Significant contribution to the prediction ($P \leq 0.05$) is indicated by "X." A tendency to contribute to the prediction ($0.05 < P \leq 0.10$) is indicated by "x."
[2]B:F = Bacteroidetes-Firmicutes ratio.

Supplementary Table S3. Summary of explanatory variables used in predictions of the microbiome[1]

Item	Bacteroidetes	Firmicutes	B:F[2]	Proteobacteria	Chao1 Richness	Shannon Diversity
Diet variables, % DM						
OM					X	
CP					X	
NDF						X
Starch					X	
EE				X		
Forage				X		
Experimental variables						
Publication year	x	X				X
Breed type	X	X	X	x		

[1]Significant contribution to the prediction ($P \leq 0.05$) is indicated by "X." A tendency to contribute to the prediction ($0.05 < P \leq 0.10$) is indicated by "x."
[2]B:F = Bacteroidetes-Firmicutes ratio.

Supplementary Table S4. Assumptions for nutrient composition of supplements

Supplement	% DM	% CP	% OM	% ash	% EE
Mineral	100	0.0	0.0	100	0.0
Vitamin	100	0.0	95	5.0	0.0
Vitamin-mineral premix	100	0.0	20	80	0.0
Ionophore	100	0.0	100	0.0	0.0
MGA[1]	100	0.0	100	0.0	0.0
Urea	100	281	100	0.0	0.0
Miscellaneous fats and oils	100	0.0	100	0.0	100

[1]Melengestrol acetate.

Supplementary Table S5. Monte Carlo cross-validation results for models of milk yield, mean +/- standard deviation

Item[1]	Diet	Microbial	Microbial + DMI	Experimental	Diet + Microbial	Diet + Experimental	Microbial + Experimental	Diet + Microbial + Experimental
Model no.	1	2	3	4	5	6	7	8
DMI, kg/d	0.536 +/- 0.18		0.708 +/- 0.20		0.659 +/- 0.32	0.596 +/- 0.23		0.340 +/- 0.21
CP, % DM	1.36 +/- 0.28				1.68 +/- 0.39	1.08 +/- 0.55		1.44 +/- 0.60
NFC, % DM						0.0972 +/- 0.083		
Bacteroidetes		-0.354 +/- 0.17	0.106 +/- 0.037					
Firmicutes		0.808 +/- 0.22	0.112 +/- 0.043				0.799 +/- 0.24	-0.684 +/- 7.0
B:F		10.0 +/- 3.0					8.10 +/- 2.4	-32.4 +/- 67
Proteobacteria		-1.45 +/- 0.70			0.160 +/- 0.17			
Chao1 richness		-0.00136 +/- 0.00050					-1.16 +/- 1.2	
Year				0.530 +/- 0.18		0.399 +/- 0.25		
Fluid fraction							58.1 +/- 20	-82.3 +/- 302
Solid fraction							19.3 +/- 21	-69.3 +/- 288
Firmicutes × Fluid fraction							-1.03 +/- 0.36	-2.59 +/- 172
Firmicutes × Solid fraction							-0.476 +/- 1.6	-11.5 +/- 73
B:F × Fluid fraction							9.58 +/- 42	30.4 +/- 67
B:F × Solid fraction								25.9 +/- 96
Fit statistics								
RMSE	13.7 +/- 3.1	18.3 +/- 4.4	13.8 +/- 2.3	27.5 +/- 5.4	14.8 +/- 3.2	14.5 +/- 2.8	26.7 +/- 980	16.1 +/- 179
uCCC	0.52 +/- 0.15	0.465 +/- 0.22	0.570 +/- 0.13	0.0186 +/- 0.036	0.443 +/- 0.21	0.508 +/- 0.15	0.030 +/- 24	0.807 +/- 95

[1]B:F = Bacteroidetes-Firmicutes ratio; RMSE = root mean square error; uCCC = uncalibrated concordance correlation coefficient.

Supplementary Table S6. Monte Carlo cross-validation results for models of dairy feed efficiency, mean +/- standard deviation[1]

Item[2]	Diet	Microbial	Experimental	Diet + Microbial	Diet + Experimental	Microbial + Experimental
Model no.	1	2	3	4	5	6
DM, %						
CP, % DM						
NDF, % DM						
ADF, % DM						
Starch, % DM				-0.000444 +/- 0.0045		
EE, % DM						
Shannon diversity		-0.0474 +/- 0.0096		-0.0508 +/- 0.022		
Year			-0.00670 +/- 0.0076			
Fluid fraction						
Solid fraction						
Shannon diversity × Fluid fraction						
Shannon diversity × Solid fraction						
Fit statistics						
RMSE		10.4 +/- 2.3	11.9 +/- 2.0	10.9 +/- 2.5		
uCCC		0.218 +/- 0.30	0.0141 +/- 0.089	0.186 +/- 0.30		

[1]Models 1, 5, and 6 possessed insufficient numbers of observations to perform cross-validation.
[2]RMSE = root mean square error; uCCC = uncalibrated concordance correlation coefficient.

Supplementary Table S7. Monte Carlo cross-validation results for models of dry matter intake, mean +/- standard deviation[1]

Item[2]	Diet	Microbial	Experimental	Diet + Microbial	Diet + Experimental	Microbial + Experimental	Diet + Microbial + Experimental
Model no.	1	2	3	4	5	6	7
DM, %							
Starch, % DM	-0.399 +/- 0.094			-0.304 +/- 0.069	-0.354 +/- 0.094		
NFC, % DM	0.254 +/- 0.11			0.297 +/- 0.070	0.189 +/- 0.10		
OM, % DM	0.418 +/- 0.15				0.345 +/- 0.20		
Forage, % DM	-0.0710 +/- 0.032				-0.0828 +/- 0.033		
B:F				-0.130 +/- 0.14			
Shannon diversity		-0.501 +/- 0.32				0.414 +/- 0.27	
Year			1.04 +/- 0.14			0.217 +/- 1.3	
Fluid fraction						5.82 +/- 2.3	
Solid fraction						5.98 +/- 2.6	
Bos indicus breed type			-0.909 +/- 7.2			-3.87 +/- 7.0	
Dairy breed type			16.6 +/- 10.1		-1.74 +/- 6.6	5.36 +/- 2.8	
Shannon diversity × Fluid fraction						-1.86 +/- 0.32	
Shannon diversity × Solid fraction						16.5 +/- 30	
Fit statistics							
RMSE	35.3 +/- 2.8	40.8 +/- 6.1	22.3 +/- 14.8	30.6 +/- 4.1	14.4 +/- 9.3	12.6 +/- 15	
uCCC	0.112 +/- 0.10	0.0578 +/- 0.10	0.557 +/- 0.097	0.0403 +/- 0.15	0.206 +/- 13.0	0.861 +/- 963	

[1]Model 7 possessed insufficient numbers of observations to perform cross-validation.
[2]B:F = Bacteroidetes-Firmicutes ratio; RMSE = root mean square error; uCCC = uncalibrated concordance correlation coefficient.

Supplementary Table S8. Monte Carlo cross-validation results for models of Bacteroidetes relative abundance, mean +/- standard deviation

Item[1]	Diet	Experimental	Diet + Experimental
Model no.	1	2	3
CP, % DM	0.615 +/- 0.45		
NDF, % DM			-0.177 +/- 0.10
Year		2.49 +/- 0.47	2.50 +/- 0.52
Bos indicus breed type		25.4 +/- 6.1	35.6 +/- 7.9
Dairy breed type		3.86 +/- 4.0	3.74 +/- 5.7
Fit statistics			
RMSE	52.0 +/- 4.9	48.4 +/- 4.1	50.1 +/- 5.1
uCCC	0.00688 +/- 0.033	0.237 +/- 0.064	0.270 +/- 173

[1]RMSE = root mean square error; uCCC = uncalibrated concordance correlation coefficient.

Supplementary Table S9. Monte Carlo cross-validation results for models of Firmicutes relative abundance, mean +/- standard deviation

Item[1]	Diet	Experimental	Diet + Experimental
Model no.	1	2	3
DM, %	-0.0770 +/- 0.057		-0.0641 +/- 0.058
Year		-3.00 +/- 0.38	-2.97 +/- 0.42
Bos indicus breed type		-28.5 +/- 3.7	-29.9 +/- 4.3
Dairy breed type		-3.54 +/- 3.1	-4.97 +/- 4.1
Fit statistics			
RMSE	50.1 +/- 3.8	41.9 +/- 3.2	43.0 +/- 3.6
uCCC	0.00312 +/- 0.026	0.446 +/- 0.066	0.423 +/- 0.073

[1]RMSE = root mean square error; uCCC = uncalibrated concordance correlation coefficient.

Supplementary Table S10. Monte Carlo cross-validation results for models of the Bacteroidetes-Firmicutes ratio, mean +/- standard deviation

Item[1]	Diet	Experimental	Diet + Experimental
Model no.	1	2	3
DM, %	0.0104 +/- 0.013		0.00916 +/- 0.012
Bos indicus breed type		9.82 +/- 5.1	9.73 +/- 5.2
Dairy breed type		-0.0422 +/- 0.59	0.182 +/- 0.78
Fit statistics			
RMSE	78.1 +/- 186	172 +/- 130	173 +/- 138
uCCC	0.00268 +/- 0.031	0.165 +/- 0.11	0.147 +/- 0.10

[1]RMSE = root mean square error; uCCC = uncalibrated concordance correlation coefficient.

Supplementary Table S11. Monte Carlo cross-validation results for models of Proteobacteria relative abundance, mean +/- standard deviation

Item[1]	Diet	Experimental	Diet + Experimental
Model no.	1	2	3
CP, % DM			-0.0636 +/- 0.14
EE, % DM	3.16 +/- 0.64		
Forage, % DM	0.0264 +/- 0.020		
Bos indicus breed type		-5.06 +/- 0.56	-6.21 +/- 0.73
Dairy breed type		-2.53 +/- 0.65	-3.85 +/- 0.77
Fit statistics			
RMSE	75.6 +/- 32	97.9 +/- 34	87.2 +/- 25
uCCC	0.260 +/- 0.26	0.174 +/- 0.092	0.314 +/- 0.13

[1]RMSE = root mean square error; uCCC = uncalibrated concordance correlation coefficient.

Supplementary Table S12. Monte Carlo cross-validation results for models of Chao1 richness, mean +/- standard deviation[1]

Item[2]	Diet	Experimental	Diet + Experimental
Model no.	1	2	3
CP, % DM	-2.10 +/- 9.8		
Starch, % DM	5.42 +/- 5.3		
OM, % DM	-1505 +/- 7.3		
Bos indicus breed type			
Dairy breed type			
Fit statistics			
RMSE	0.998		
uCCC	0.69		

[1]Models 2 and 3 possessed insufficient numbers of observations to perform cross-validation.
[2]RMSE = root mean square error; uCCC = uncalibrated concordance correlation coefficient.

Supplementary Table S13. Monte Carlo cross-validation results for models of Shannon diversity, mean +/- standard deviation

Item[1]	Diet	Experimental	Diet + Experimental
Model no.	1	2	3
NDF, % DM	0.0339 +/- 0.30		0.0343 +/- 0.0066
Year		0.436 +/- 0.032	
Bos indicus breed type		0.441 +/- 1.2	0.253 +/- 1.1
Dairy breed type		0.313 +/- 1.2	0.714 +/- 2.0
Fit statistics			
RMSE	44.5 +/- 4.8	41.3 +/- 3.9	44.3 +/- 5.0
uCCC	0.0783 +/- 0.049	0.234 +/- 29	0.0926 +/- 0.20

[1]RMSE = root mean square error; uCCC = uncalibrated concordance correlation coefficient.

CHAPTER 6: Characterizing Effects of Ingredients Differing in Ruminally Degradable Protein and Fiber Supplies on the Rumen Microbiome Using Next-Generation Sequencing

This chapter will be published as: Gleason, C. B., R. E. Settlage, L. M. Beckett, and R. R. White. Characterizing effects of ingredients differing in ruminally degradable protein and fiber supplies on the rumen microbiome using Next-Generation sequencing. Journal of Animal Science.

ABSTRACT

The ratio of concentrate to forage within diets is known to alter rumen microbial profiles, but comparatively less information is available on the effect of differing degradabilities of individual nutrients on the microbiome. The objective of this study was to investigate rumen microbial responses to diets composed of protein and fiber sources expected to vary in nutrient degradability. The responses of interest included relative abundances of bacterial taxa as well as estimations of community richness and diversity. Ten ruminally cannulated wethers (Suffolk, Dorset, or Suffolk x Dorset) received 4 diet treatments consisting of either beet pulp or timothy hay and soybean meal (**SBM**) or heat-treated soybean meal (**HSBM**) in a partially replicated 4 x 4 Latin square experiment for 21 d. Timothy hay and beet pulp were expected to provide differing rumen degradabilities of neutral detergent fiber (**NDF**) while the soybean meals were expected to provide differing rumen degradabilities of crude protein (**CP**). Solid and liquid samples of rumen contents were collected for microbial DNA isolation and Next-Generation sequencing. Numerous rumen bacterial population shifts were observed due to change in fiber source, with increased abundances ($P < 0.05$) of fibrolytic populations associated with timothy hay diets compared with beet pulp diets. Conversely, populations of the pectin-degrading genera, *Treponema* and *Lachnospira*, increased on the beet pulp treatment ($P = 0.015$ and $P = 0.0049$, respectively). Limited impact on bacterial taxa was observed between diets differing in protein source. The *Paraprevotellaceae* genus YRC22 was observed to increase in abundance on HSBM diets ($P = 0.023$) and the phylum Spirochaetes tended to be more abundant on SBM than HSBM diets ($P = 0.071$). Beet pulp decreased rumen bacterial diversity ($P = 0.0027$) and tended to decrease bacterial species richness ($P = 0.051$) compared to timothy hay. Our results serve to

further underscore the sensitivity of rumen microbes to changes in their preferred substrates, particularly of those associated with fiber degradation.

Keywords: Next-Generation sequencing, nutrients, rumen microbiome, sheep

INTRODUCTION

The rumen ecosystem is composed of diverse and dynamic populations of microorganisms, including bacteria, fungi, and protozoa, that are responsible for the vast majority of feed digestion by the host animal (Hungate et al., 1964; Krause et al., 2013; Jewell et al., 2015). The energy harvested from volatile fatty acids (**VFA**) produced through microbial fermentation of plant material is estimated at 70% of the total metabolic energy used by the ruminant (Bergman, 1990). In addition, 60 to 85% of amino acids reaching the small intestine are supplied by rumen microbial protein synthesis (Storm et al., 1983). The rumen microbiome therefore serves as an important intermediate between diet and animal performance (Mullins et al., 2013; Gleason and White, 2018).

High-throughput or Next-Generation sequencing technologies, such as Illumina, have propelled investigations of the rumen microbiome forward with their ability to rapidly and economically sequence the bacterial 16S rRNA gene. Along with bioinformatics techniques and pipelines, Next-Generation sequencing has allowed for detailed characterization of the microbiome, which has greatly enhanced our understanding of this complex ecosystem (Lima et al., 2015). Common targets of investigation include relative abundances of the major bacterial taxa, in addition to estimates of species richness and diversity. Variations in these measurements have been linked to variations in livestock production traits, including milk yield and components (Jami et al., 2014; Lima et al., 2015; Indugu et al., 2017), average daily gain (Hernandez-Sanabria et al., 2012; Myer et al., 2015), and feed efficiency (Hernandez-Sanabria et al., 2010; Carberry et al., 2012; Jewell et al., 2015).

Previous research has demonstrated that the microbial community can be influenced by diet forage-to-concentrate ratio (de Menezes et al., 2011; Carberry et al., 2012; Petri et al., 2013) and by alterations in amounts of specific concentrate ingredients (Callaway et al., 2010; Petri et al., 2014). However, little information on the effects of differing nutrient degradabilities is currently available. Therefore, our objective was to examine how the rumen microbiome responds to supplies of protein and fiber sources possessing differing degradability profiles of crude protein (**CP**) and neutral detergent fiber (**NDF**), respectively.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

All animal use and procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (Protocol #18-096). Ten ruminally cannulated commercial wethers (Suffolk, Dorset or Suffolk x Dorset) were housed in individual stalls at the Smithfield Farm, Virginia Tech, Blacksburg, VA. Wethers were approximately 1.5 years of age and weighed an average of 62.1 ± 6.6 kg at trial commencement. Wethers were assigned to treatments in a partially replicated 4 x 4 Latin square so that treatment groups were balanced for initial body weight with 2 wethers serving as alternates. Treatments were assigned using a 2 x 2 factorial approach and included feedstuffs intended to supply varying rumen degradabilities of CP and NDF. Soybean meal (**SBM**) and heat-treated soybean meal (**HSBM**) were utilized as the CP sources with high and low rumen degradabilities, respectively. These meals were pelleted with alfalfa, corn, barley, wheat middlings, trace mineral salt, and a sheep vitamin premix (Table 1). Pelleted beet pulp (**BP**) and long timothy hay (**TH**) represented the NDF sources, with BP expected to undergo a faster rate of fiber degradation compared to TH. Diets were prepared by

combining the appropriate protein pellet with the appropriate fiber source to create the 4 treatments: highly degradable CP plus lowly degradable NDF (**SBM-TH**), highly degradable CP plus highly degradable NDF (**SBM-BP**), lowly degradable CP plus lowly degradable NDF (**HSBM-TH**), and lowly degradable CP plus highly degradable NDF (**HSBM-BP**). Each animal consumed each of the 4 diets for 21 d. Gradual diet adaptation occurred during the first 3 d and 100% of the diet treatment was offered for the remaining 18 d. Animals were fed once daily at 0800 h from d 0 to 15 then, from d 16 to 21, animals were fed 1/12th of their daily ration every 2 h in an effort to more closely mimic a metabolic steady state. This was done because animals received stable isotope infusions of VFA from days 17 to 20 for a separate investigation into rumen VFA dynamics (Gleason, et al., *unpublished*). Clean, fresh water was available at all times.

Sample Collection and DNA Isolation

Fluid and solid samples of rumen contents from all animals were collected at 0800 h on day 16 of each period. Contents were collected via the cannula from the dorsal sac after slight hand mixing and strained through 1 layer of gauze to separate the fluid and solid fractions. Samples were stored in cryovials at -80°C until total DNA extraction. Extraction was performed on 0.5 g of a liquid or solid sample using a QIAamp DNA stool minikit (Qiagen, Valencia, CA) following the manufacturer's protocol. Sample DNA concentrations and 260/280 ratios were obtained using a spectrophotometer (Epoch2 Microplate Reader, Biotek, Winooski, VT). Extracted DNA was stored at -80°C until further processing.

PCR Amplification, Next-Generation Sequencing, and Bioinformatic Analysis

The universal primers 515F and 926R were chosen to amplify the V4–V5 region of the 16S rRNA gene following the Earth Microbiome Project protocol (https://www.earthmicrobiome.org/). The PCR reaction mixture included 13.0 µL of PCR-grade water, 1.0 µL of template DNA, 10 µM of each primer, and 10.0 µL of 5PRIME HotMasterMix (2x) (Quantabio, Beverly, MA). Samples were amplified in duplicate under the following thermocycler conditions: 94°C for 3 min for initial denaturing, then 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s. A final elongation step occurred at 72°C for 10 min followed by a hold at 4°C. After pooling duplicates, all amplicons were visualized on a 2% agarose gel and quantitated on a Qubit fluorometer (Fisher Scientific, Hampton, NH). Normalization was performed based on Qubit results, and amplicons were purified on a Pippin Prep (Sage Science, Beverly, MA) targeting a 520bp range. Quantitative PCR was then run on the pool. Amplicons were loaded at 9.5 pM and sequenced using the MiSeq v3 600-cycle kit on the Miseq platform (Illumina, Inc., San Diego, CA). All sequence analyses were conducted using the QIIME 2 bioinformatics platform version 2019.10 (Bolyen et al., 2019) following the protocol of Estaki et al. (2020). Sequence quality filtering and denoising was performed using Deblur (Amir et al., 2017). Sequences were then clustered into OTUs and taxonomic identities assigned at a 97% identity cut-off using a naive Bayes classifier with the Greengenes database as a reference (DeSantis et al., 2006). Relative abundances of taxa were obtained by dividing the reads assigned to a given taxon by the total number of reads present. Richness and diversity estimates were obtained using the Core Diversity command.

Statistical Analysis

Data was analyzed using the nlme package (Pinheiro et al., 2020) in R version 3.6.1 (R Core Team, 2019). Response variables included percent relative abundances of bacterial taxa at the phylum, family, and genus levels, number of observed OTUs, and Shannon diversity index value. Response variables were analyzed using the linear model:

$Y_{ijkl} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + c_k + d_l + e_{ijkl}$,

where μ represents the overall mean, α_i is the effect of the i^{th} CP source, β_j is the effect of the j^{th} NDF source, $\alpha\beta_{ij}$ is the interaction of CP source i and NDF source j, c_k represents the random effect of animal k, d_l represents the random effect of period l, and e_{ijkl} is the residual error associated with CP source i, NDF source j, animal k, and period l. Compound Symmetry, Unstructured, and 1st Order Autoregressive residual error variance structures were compared for each response variable and the Akaike information criterion (**AIC**) was used to assess model quality (Hurvich and Tsai, 1989). The model with the lowest AIC value was subjected to ANOVA and least square means were determined. Statistical significance was considered when $P < 0.05$ and a tendency considered when $0.05 \leq P < 0.10$.

RESULTS AND DISCUSSION

Relative abundances of bacterial taxa identified in the rumen fluid and solid fractions are located in Tables 2 and 3, respectively. Figures 1a-3b depict compositions of the bacterial communities on the diet treatments at different taxonomic levels. Alpha diversity metrics of the bacterial communities associated with rumen fluid and solid fractions are presented in Table 4.

Demultiplexed sequence counts totaled 14,851,796 reads with a median length of 300 nucleotides and an average of 198,024 sequences per sample. The predominant bacterial phylum

identified was Bacteroidetes, ranging between 73.1 and 78.5% relative abundance across treatment means in rumen fluid (Table 2) and between 52.4 and 74.5% in rumen solids (Table 3). Other phyla representing at least 1% mean relative abundance included Firmicutes, Fibrobacteres, Proteobacteria, Spirochaetes, Tenericutes, and Cyanobacteria in both fractions. Populations of Fibrobacteres and Spirochaetes were considerably more pronounced in the solid fraction than in fluid (Figures 1a and 1b), which is consistent with previous observations (de Menezes et al., 2011). A number of taxonomic lines were found to display variation in response to the different diet treatments. These belonged largely to the predominant phyla identified, including Bacteroidetes, Firmicutes, Spirochaetes, Tenericutes, and Verrucomicrobia. Increases in relative abundance were mainly associated with the TH treatment and decreases with the BP treatment, with few exceptions. The majority of microbial variation due to fiber source was identified in the solid ruminal fraction compared to the fluid fraction. This was unsurprising given that fibrolytic activity would be most prominent in the fiber mat of the rumen.

Bacterial Population Shifts Associated with Fiber Source

Bacteroidetes and its family S24-7 were found to increase ($P = 0.014$ and $P = 0.0050$, respectively; Table 3) in animals receiving the TH treatment compared to those on the BP treatment. Two additional Bacteroidetes families, *Bacteroidaceae* and *Prevotellaceae*, tended to be more prominent in response to the TH treatment ($P = 0.089$ and $P = 0.080$, respectively; Table 3) as well as two genera, BF311 ($P = 0.092$; Table 3) and YRC22 ($P = 0.052$; Table 2). Fibrolytic activities are abundant within the Bacteroidetes phylum (Terrapon et al., 2015). Because Bacteroidetes is generally the most prominent phylum in the rumen and cellulose is the main energy source of ruminants (Ensminger et al., 1990), it is logical that this bacterial group would be sensitive to changes in the fiber substrate provided. The Bacteroidetes families

Bacteroidaceae, *Prevotellaceae* and S24-7 are known to be involved in digestion of fiber (Lan et al., 2006; Ivarsson et al., 2014; Gamage et al., 2018). Even though the *Bacteroidaceae* genus BF311's substrate preferences have yet to be fully characterized (Bi et al., 2018), observations of increased abundance in goats when hay was fed also supports an involvement in forage fiber degradation (Zhang et al., 2018). The same experiment reported similar behavior of YRC22 (Zhang et al., 2018). Our observations of fiber effects on Bacteroidetes and its family S24-7, as well as tendencies for *Prevotellaceae*, *Bacteroidaceae*, BF311, and YRC22 to be affected by fiber source may reflect their known (or probable) functional roles and indicate sensitivity to changes in their substrate.

Firmicutes, like Bacteroidetes, was more abundant on the TH treatment than on BP (P = 0.0054; Table 3). This fiber effect was seen in its *Clostridiaceae* and *Veillonellaceae* families (P = 0.0094 and P = 0.016, respectively; Table 3) and tended to impact *Erysipelotrichaceae* and *Ruminococcaceae* populations as well (P = 0.088 and P = 0.064, respectively; Table 3). Firmicutes genera displaying decreased abundance ($P < 0.05$; Tables 2 and 3) in response to BP treatment compared to TH included *Acidaminococcus*, *Clostridium*, *Oscillospira*, *Ruminococcus*, and *Succiniclasticum*. *Ruminococcus* and *Clostridium* have been established as prominent fiber digesters in the rumen (Preston and Leng, 1987; Chesson and Forsberg, 1997). *Oscillospira* and *Succiniclasticum* are also cellulolytic and have been shown to increase as the dietary forage-to-concentrate ratio increases (Mackie et al., 2003; Han et al., 2019). Additionally, fiber has been shown to stimulate *Acidaminococcus* populations *in vitro* (Gong et al., 2019). Although we only observed a tendency of *Erysipelotrichaceae* to increase on TH, previous research has demonstrated greater prominence of this family in cattle consuming hay as a forage source

compared to corn silage or grass silage (Deusch et al., 2017). Our results serve to further underscore the sensitivity of these taxa to changes in their fiber substrate.

Population decreases associated with BP were also observed in Verrucomicrobia and its family RFP12 ($P = 0.037$ and $P = 0.037$, respectively; Table 2) and in the Tenericutes family *Anaeroplasmataceae* and its genus *Anaeroplasma* ($P = 0.020$ and $P = 0.0081$, respectively; Table 3). Tenericutes itself, along with the family *Coriobacteriaceae*, and *Coriobacteriaceae's* genus *Olsenella* tended to be less abundant on BP compared to the TH treatment ($P = 0.083$, $P = 0.089$, and $P = 0.085$, respectively; Table 3). *Methanomassiliicoccaceae*, the sole Archaeal family in our report, also exhibited a population decline when BP diets were fed ($P = 0.021$; Table 3). Our observations were largely consistent with those in the literature. RFP12 has been shown to decrease in abundance as dietary forage content is decreased (Plaizier et al., 2017). *Anaeroplasma* has been correlated with fiber digestion (Niu et al., 2015), and *Olsenella* has been suggested to participate prominently in the degradation of ryegrass (Huws et al., 2016). Our results indicate that these taxa are more strongly associated with the degradation of cellulose and hemicellulose in forages than with the degradation of soluble fiber in beet pulp. The behavior of *Methanomassiliicoccaceae* in response to forage or fiber content appears to be less clear. This family has been reported to decrease in abundance when dietary forage level was reduced (Zhu et al., 2018), but has also been observed to increase as forage fiber was replaced with wheat bran and soy hulls as non-forage fiber sources (Wang et al., 2018), in direct contradiction to our findings. Additional work is necessary to fully characterize the effects of forage fiber versus non-forage fiber on *Methanomassiliicoccaceae*.

Contrasting with the previous taxa mentioned above, the phylum Spirochaetes was approximately 3 to 4 times more abundant in animals on the BP treatment compared to those

receiving TH (P = 0.013; Table 3). Its family *Spirochaetaceae* and genus *Treponema* reflected this effect as well (P = 0.015). The Firmicutes genus *Lachnospira* was the only other taxonomic group displaying a population increase in association with the BP treatment rather than a decrease (P = 0.0049; Table 3). This response is most likely due to the high pectin content of beet pulp (Codling and Woodman, 1929). *Treponema* and *Lachnospira* are both known to be involved in pectin degradation (Wojciechowicz and Ziołecki, 1979; Kasperowicz, 1994). Previous research has also demonstrated increased abundances of these genera in response to stimulation by pectin (Liu et al., 2015; Bang et al., 2018), and according to Liu and colleagues (2015), ruminal *Treponema* populations were "remarkably supported" by the provision of pectin. The positive responses we observed in these taxa provide further evidence of their sensitivity to pectin content and broadly underscore the significance of substrate availability in influencing microbial population shifts.

Numerous investigations have evaluated the effects of hay inclusion on the rumen microbial community within the context of comparing different forage-to-concentrate ratios (Fernando et al., 2010; Hook et al., 2011; Klevenhusen et al., 2017; Zhang et al., 2017). The recurring theme throughout these studies is that fibrolytic bacterial populations increase when forage content is increased, in agreement with our observations. Comparatively less research attention, however, has been dedicated to rumen microbial changes on beet pulp-based diets and to our knowledge, no studies have utilized BP as a replacement for forage in the diet and compared differences in the microbial profiles. Studies have instead focused on using BP as a high-fiber/low-starch energy source to replace starchy concentrate feeds (Zhao et al., 2013; Münnich et al., 2018). Nonetheless, these studies have still observed reductions in fiber-degrading bacterial populations in response to BP treatment, which is consistent with our

findings (Zhao et al., 2013; Münnich et al., 2018). These results suggest that the optimal type of fiber substrate likely differs between various bacterial populations involved in its breakdown and that fibrolytic species may not be supported as adequately by non-forage fiber sources. This highlights the need for future ruminant nutrition work to move away from broad classification of nutrients and towards characterizing specific substrate supplies.

Bacterial Population Shifts Associated with Protein Source

In contrast to the microbial responses associated with fiber type, very little microbial variation was observed when protein source was varied. Major taxa associated with ruminal protein degradation, including *Butyrivibrio*, *Prevotella*, and *Ruminobacter* (Mackie et al., 2001; McDonald et al., 2011) displayed similar relative abundances across diet treatments, appearing to indicate no sensitivity to the provision of heat-treated versus non-treated SBM. However, the phylum Spirochaetes did tend to be more abundant on SBM than HSBM ($P = 0.071$; Table 2). The *Paraprevotellaceae* genus YRC22 was observed to increase in abundance on HSBM diets, and this effect was consistent both in the fluid fraction ($P = 0.0072$, Table 2) and solid fraction ($P = 0.023$; Table 3). These responses may be evidence of involvement in protein metabolism. Some members of Spirochaetes have been observed to produce enzymes with roles in peptide degradation, including trypsin, chymotrypsin, and arylamidases (Nordhoff et al., 2005; Newbrook et al., 2017). Because our observed shift in Spirochaetes was quite minimal, however, more research is necessary to clarify the sensitivity of this phylum to changes in protein source. YRC22 has been described as a minor genus with relatively unknown function (Zhu et al 2018). Previous research has identified a strong correlation between YRC22 abundance and concentrations of certain amino acids in rumen fluid, leading investigators to conclude this genus may be involved in their metabolism to some degree (Hua et al., 2017). The sensitivity of

YRC22 to changes in protein source that we observed provides additional evidence of a potential link with the processing or utilization of protein and, along with its response to our fiber treatments, underscores the need for further investigation of this genus.

As explained above, our protein treatments were represented by SBM and heated SBM. Treating SBM with heat leads to denaturation of proteins, which may decrease their solubility and slow their ruminal degradation rate (Russell et al., 1992). Decreased ruminal availability of SBM protein due to heat treatment is well documented in both cattle (Plegge et al., 1985; Ljøkjel et al., 2000; Borucki Castro et al., 2007) and sheep (Glimp et al., 1967; Demjanec et al., 1995), but very little research has investigated the impacts on rumen microbial populations. Plegge et al. (1985) reported that heat treatment of SBM did not impact rumen bacterial protein synthesis efficiency or bacterial nitrogen flow, but no information of the effects on the microbial populations themselves was available. Investigators evaluating the substitution of SBM with dried distillers grains with solubles (**DDGS**) observed no rumen bacterial population changes in cattle (Castillo-Lopez et al., 2017) or sheep (Shen et al., 2020). Although this protein source substitution differed from our experiment, it bears the similarity that DDGS possesses a higher proportion of rumen-undegradable protein (**RUP**) to rumen-degradable protein (**RDP**) compared to SBM (NRC, 2016). The results of these studies and ours indicate that the rumen microbiome is resistant to change in terms of population dynamics due to heat treating SBM or substituting it with another protein feed. Additionally, the high amount of CP present in our rations made it unlikely that the animals were deficient in RDP. This provides another reason for the lack of sensitivity to protein source that we observed.

Overall, our investigation was unable to detect widespread change in bacterial population dynamics due to protein source. A similar observation was made by Gong et al. (2019), who

reported that gut microbial growth patterns displayed a weaker correlation with protein than with carbohydrates. Aside from the likely absence of RDP-deficient conditions in our study, the limited overall effect of protein source on the rumen microbial community compared to fiber source may be due to the fact that plant fiber is the main nutrient source that the rumen microbiome has evolved to process and that vast numbers of rumen bacterial taxa are directly involved in fibrolytic activities (Hobson and Stewart, 1997). Additionally, rumen bacteria have the capability of utilizing recycled urea as a source of nitrogen for synthesizing their own microbial proteins (McDonald et al., 2011). It is therefore possible that the microbiome is comparatively less sensitive to changes in dietary protein solubility than it is to variations in fiber characteristics.

Microbial Richness, Diversity, and Links with Productive Efficiency

Richness and diversity of the rumen microbiome varied with fiber source, but not protein source. Shannon diversity of the solid fraction samples was greater in animals consuming TH compared to BP ($P = 0.0027$; Table 4). Similarly, the number of OTUs identified tended to increase on TH diets compared to BP diets ($P = 0.051$; Table 4).

The number of OTUs is an indication of species richness of the microbiome whereas Shannon diversity is a measure of species representation. Previous reports have found that richness and diversity may be sensitive to diet forage-to-concentrate ratio, with richness and diversity greatest on forage diets and lowest at high levels of concentrate feeding (Petri et al., 2013; Zhang et al., 2017). Our results indicate that the type of fiber source, rather than just fiber concentrations, may also influence these metrics because we balanced our experimental diets to have similar concentrations of NDF. The decline in Shannon diversity and the tendency for the

number of OTUs to decline with BP treatment also appears to mirror the bacterial population decreases associated with the BP diets discussed above.

Lower levels of richness and diversity have been linked with increased feed efficiency and reduced methane production in cattle (Shabat et al., 2016). According to Shabat (2016), efficient rumen microbiomes are composed of a smaller number of more dominant taxa that utilize a narrower range of metabolic pathways and produce metabolites that are more closely aligned with the energetic needs of the host. Less efficient microbiomes can be characterized by more diverse populations that employ a wide range of metabolic pathways, some of them resulting in greater energy loss, such as through methane production (Shabat et al., 2016). Methane represents wasted dietary energy for the animal as it cannot be absorbed and also contributes to agricultural greenhouse gas emissions (Johnson and Ward, 1996). The reduction in abundance of the methanogen family *Methanomassiliicoccaceae* that we observed may be further evidence that substituting TH with BP could potentially shift the microbiome in a more energetically efficient direction. This family is poorly characterized, however, and additional research is necessary to clarify its contribution to ruminant methane production (Huang et al., 2016). Two bacterial taxa that were also observed to decrease in abundance on BP diets, *Clostridium* and *Succiniclasticum*, have been previously linked with poor feed efficiency in growing steers (Hernandez-Sanabria et al., 2012; Myer et al., 2015). Because we utilized mature wethers in our experiment and measuring emissions was outside our study scope, further work will be needed to confirm if the taxonomic shifts we observed would result in improved growth efficiency and lower methane production.

Conclusions

In this study, we sought to explore rumen microbial responses to differing dietary sources of fiber and protein and, in doing so, further the understanding of the complex relationship between the rumen microbiome and diet. Fibrolytic bacterial populations and overall microbial diversity were found to decrease when beet pulp was utilized as a non-forage fiber alternative to timothy hay whereas pectin degraders were promoted. While less taxa exhibited sensitivity to differences in protein source, those that did have been reported as possessing links to proteolytic activities. Nutrient source, rather than simply nutrient concentration, may therefore exert a marked impact on bacterial population dynamics in the ovine rumen microbiome. Additional research is required to determine if the differing nutrient sources utilized in our study and their subsequent influence on the rumen microbiome may translate into biologically and economically relevant alterations in livestock performance.

LITERATURE CITED

Amir, A., D. McDonald, J. A. Navas-Molina, E. Kopylova, J. T. Morton, Z. Zech Xu, E. P. Kightley, L. R. Thompson, E. R. Hyde, A. Gonzalez, and R. Knight. 2017. Deblur rapidly resolves single-nucleotide community sequence patterns. mSystems. 2:e00191-16. doi:10.1128/mSystems.00191-16.

Bang, S.-J., G. Kim, M. Y. Lim, E.-J. Song, D.-H. Jung, J.-S. Kum, Y.-D. Nam, C.-S. Park, and D.-H. Seo. 2018. The influence of in vitro pectin fermentation on the human fecal microbiome. AMB Express. 8:98. doi:10.1186/s13568-018-0629-9.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70:567–590. doi:10.1152/physrev.1990.70.2.567.

Bi, Y., S. Zeng, R. Zhang, Q. Diao, and Y. Tu. 2018. Effects of dietary energy levels on rumen bacterial community composition in Holstein heifers under the same forage to concentrate ratio condition. BMC Microbiol. 18:69. doi:10.1186/s12866-018-1213-9.

Bolyen, E., J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander, E. J. Alm, M. Arumugam, and F. Asnicar. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37:852–857. doi:10.1038/s41587-019-0209-9.

Borucki Castro, S. I., L. E. Phillip, H. Lapierre, P. W. Jardon, and R. Berthiaume. 2007. Ruminal degradability and intestinal digestibility of protein and amino acids in treated soybean meal products. J. Dairy Sci. 90:810–822. doi:10.3168/jds.S0022-0302(07)71565-5.

Callaway, T. R., S. E. Dowd, T. S. Edrington, R. C. Anderson, N. Krueger, N. Bauer, P. J. Kononoff, and D. J. Nisbet. 2010. Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. J. Anim. Sci. 88:3977–3983. doi:10.2527/jas.2010-2900.

Carberry, C. A., D. A. Kenny, S. Han, M. S. McCabe, and S. M. Waters. 2012. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. Appl. Environ. Microbiol. 78:4949–4958. doi:10.1128/AEM.07759-11.

Castillo-Lopez, E., C. J. R. Jenkins, N. D. Aluthge, W. Tom, P. J. Kononoff, and S. C. Fernando. 2017. The effect of regular or reduced-fat distillers grains with solubles on rumen methanogenesis and the rumen bacterial community. J. Appl. Microbiol. 123:1381–1395. doi:10.1111/jam.13583.

Chesson, A., and C. W. Forsberg. 1997. Polysaccharide degradation by rumen microorganisms. In: The rumen microbial ecosystem. Chapman and Hall, London. p. 329–381.

Codling, A. J., and H. E. Woodman. 1929. Sugar-beet pulp as a source of pectin. J. Agric. Sci. 19:701–714. doi:10.1017/S0021859600013411.

Demjanec, B., N. R. Merchen, J. D. Cremin, C. G. Aldrich, and L. L. Berger. 1995. Effect of roasting on site and extent of digestion of soybean meal by sheep: I. Digestion of nitrogen and amino acids. J. Anim. Sci. 73:824–834. doi:10.2527/1995.733824x.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72:5069–5072. doi:10.1128/AEM.03006-05.

Deusch, S., A. Camarinha-Silva, J. Conrad, U. Beifuss, M. Rodehutscord, and J. Seifert. 2017. A structural and functional elucidation of the rumen microbiome influenced by various diets and microenvironments. Front. Microbiol. 8:1605. doi:10.3389/fmicb.2017.01605.

Ensminger, M. E., J. E. Oldfield, and W. W. Heinemann. 1990. Feeds and nutrition. 2nd ed. The Ensminger Publishing Company, Clovis, CA.

Estaki, M., L. Jiang, N. A. Bokulich, D. McDonald, A. González, T. Kosciolek, C. Martino, Q. Zhu, A. Birmingham, Y. Vázquez-Baeza, M. R. Dillon, E. Bolyen, J. G. Caporaso, and R. Knight. 2020. QIIME 2 enables comprehensive end-to-end analysis of diverse microbiome data and comparative studies with publicly available data. Curr. Protoc. Bioinforma. 70. doi:10.1002/cpbi.100.

Fernando, S. C., H. T. Purvis, F. Z. Najar, L. O. Sukharnikov, C. R. Krehbiel, T. G. Nagaraja, B. A. Roe, and U. DeSilva. 2010. Rumen microbial population dynamics during adaptation to a high-grain diet. Appl. Environ. Microbiol. 76:7482–7490. doi:10.1128/AEM.00388-10.

Gamage, H. K. A. H., S. G. Tetu, R. W. W. Chong, D. Bucio-Noble, C. P. Rosewarne, L. Kautto, M. S. Ball, M. P. Molloy, N. H. Packer, and I. T. Paulsen. 2018. Fiber supplements derived from sugarcane stem, wheat dextrin and Psyllium husk have different in vitro effects on the human gut microbiota. Front. Microbiol. 9:1618. doi:10.3389/fmicb.2018.01618.

Gleason, C. B., and R. R. White. 2018. Variation in animal performance explained by the rumen microbiome or by diet composition. J. Anim. Sci. 96:4658–4673. doi:10.1093/jas/sky332.

Glimp, H. A., M. R. Karr, C. O. Little, P. G. Woolfolk, G. E. Mitchell, and L. W. Hudson. 1967. Effect of reducing soybean protein solubility by dry heat on the protein utilization of young lambs. J. Anim. Sci. 26:858–861. doi:10.2527/jas1967.264858x.

Gong, L., H. Chi, J. Wang, H. Zhang, and B. Sun. 2019. In vitro fermentabilities of whole wheat as compared with refined wheat in different cultivars. J. Funct. Foods. 52:505–515. doi:10.1016/j.jff.2018.11.027.

Han, X., B. Li, X. Wang, Y. Chen, and Y. Yang. 2019. Effect of dietary concentrate to forage ratios on ruminal bacterial and anaerobic fungal populations of cashmere goats. Anaerobe. 59:118–125. doi:10.1016/j.anaerobe.2019.06.010.

Hernandez-Sanabria, E., L. A. Goonewardene, M. Li, D. F. Mujibi, P. Stothard, S. S. Moore, and M. C. Leon-Quintero. 2010. Linkage of particular bacterial PCR-DGGE patterns to bovine

ruminal fermentation parameters and feed efficiency traits. Appl. Environ. Microbiol. doi:0.1128/AEM.01052-10.

Hernandez-Sanabria, E., L. A. Goonewardene, Z. Wang, O. N. Durunna, and S. S. Moore. 2012. Impact of feed efficiency and diet on adaptive variations in the bacterial community in the rumen fluid of cattle. Appl. Environ. Microbiol. 78:1203–1214. doi:10.1128/AEM.05114-11.

Hobson, P. N., and C. S. Stewart. 1997. The Rumen Microbial Ecosystem. Chapman & Hall, London. Available from: https://doi.org/10.1007/978-94-009-1453-7

Hook, S. E., M. A. Steele, K. S. Northwood, J. Dijkstra, J. France, A.-D. G. Wright, and B. W. McBride. 2011. Impact of subacute ruminal acidosis (SARA) adaptation and recovery on the density and diversity of bacteria in the rumen of dairy cows. FEMS Microbiol. Ecol. 78:275–284. doi:10.1111/j.1574-6941.2011.01154.x.

Hua, C., J. Tian, P. Tian, R. Cong, Y. Luo, Y. Geng, S. Tao, Y. Ni, and R. Zhao. 2017. Feeding a high concentration diet induces unhealthy alterations in the composition and metabolism of ruminal microbiota and host response in a goat model. Front. Microbiol. 8. doi:10.3389/fmicb.2017.00138. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00138/full

Huang, X. D., G. Martinez-Fernandez, J. Padmanabha, R. Long, S. E. Denman, and C. S. McSweeney. 2016. Methanogen diversity in indigenous and introduced ruminant species on the Tibetan Plateau. Archaea. 2016:1–10. doi:10.1155/2016/5916067.

Hungate, R. E., M. P. Bryant, and R. A. Mah. 1964. The rumen bacteria and protozoa. Annu. Rev. Microbiol. 18:131–166. doi:10.1146/annurev.mi.18.100164.001023.

Hurvich, C. M., and C.-L. Tsai. 1989. Regression and time series model selection in small samples. Biometrika. 76:297–307. doi:10.1093/biomet/76.2.297.

Huws, S. A., J. E. Edwards, C. J. Creevey, P. Rees Stevens, W. Lin, S. E. Girdwood, J. A. Pachebat, and A. H. Kingston-Smith. 2016. Temporal dynamics of the metabolically active rumen bacteria colonizing fresh perennial ryegrass. J. Marchesi, editor. FEMS Microbiol. Ecol. 92:fiv137. doi:10.1093/femsec/fiv137.

Indugu, N., B. Vecchiarelli, L. D. Baker, J. D. Ferguson, J. K. P. Vanamala, and D. W. Pitta. 2017. Comparison of rumen bacterial communities in dairy herds of different production. BMC Microbiol. 17:190. doi:10.1186/s12866-017-1098-z.

Ivarsson, E., S. Roos, H. Y. Liu, and J. E. Lindberg. 2014. Fermentable non-starch polysaccharides increases the abundance of Bacteroides–Prevotella–Porphyromonas in ileal microbial community of growing pigs. Animal. 8:1777–1787. doi:10.1017/S1751731114001827.

Jami, E., B. A. White, and I. Mizrahi. 2014. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. PloS One. 9:e85423. doi:10.1371/journal.pone.0085423.

Jewell, K. A., C. A. McCormick, C. L. Odt, P. J. Weimer, and G. Suen. 2015. Ruminal bacterial community composition in dairy cows is dynamic over the course of two lactations and correlates with feed efficiency. Appl. Environ. Microbiol. 81:4697–4710. doi:10.1128/AEM.00720-15.

Johnson, D. E., and G. M. Ward. 1996. Estimates of animal methane emissions. Environ. Monit. Assess. 42:133–141. doi:10.1007/BF00394046.

Kasperowicz, A. 1994. Comparison of utilization of pectins from various sources by pure cultures of pectinolytic rumen bacteria and mixed cultures of rumen microorganisms. Acta Microbiol. Pol. 43:47–56.

Klevenhusen, F., R. M. Petri, M.-T. Kleefisch, R. Khiaosa-ard, B. U. Metzler-Zebeli, and Q. Zebeli. 2017. Changes in fibre-adherent and fluid-associated microbial communities and fermentation profiles in the rumen of cattle fed diets differing in hay quality and concentrate amount. FEMS Microbiol. Ecol. 93. doi:10.1093/femsec/fix100. Available from: http://academic.oup.com/femsec/article/doi/10.1093/femsec/fix100/4067812/Changes-in-fibreadherent-and-fluidassociated

Krause, D. O., T. G. Nagaraja, A. D. G. Wright, and T. R. Callaway. 2013. Board-invited review: Rumen microbiology: Leading the way in microbial ecology. J. Anim. Sci. 91:331–341. doi:10.2527/jas.2012-5567.

Lan, P. T. N., M. Sakamoto, S. Sakata, and Y. Benno. 2006. Bacteroides barnesiae sp. nov., Bacteroides salanitronis sp. nov. and Bacteroides gallinarum sp. nov., isolated from chicken caecum. Int. J. Syst. Evol. Microbiol. 56:2853–2859. doi:10.1099/ijs.0.64517-0.

Lima, F. S., G. Oikonomou, S. F. Lima, M. L. Bicalho, E. K. Ganda, J. C. de Oliveira Filho, G. Lorenzo, P. Trojacanec, and R. C. Bicalho. 2015. Prepartum and postpartum rumen fluid microbiomes: Characterization and correlation with production traits in dairy cows. Appl. Environ. Microbiol. 81:1327–1337. doi:10.1128/AEM.03138-14.

Liu, J., Y.-Y. Pu, Q. Xie, J.-K. Wang, and J.-X. Liu. 2015. Pectin induces an in vitro rumen microbial population shift attributed to the pectinolytic Treponema group. Curr. Microbiol. 70:67–74. doi:10.1007/s00284-014-0672-y.

Ljøkjel, K., O. M. Harstad, and A. Skrede. 2000. Effect of heat treatment of soybean meal and fish meal on amino acid digestibility in mink and dairy cows. Anim. Feed Sci. Technol. 84:83–95. doi:10.1016/S0377-8401(00)00104-8.

Mackie, R. I., R. I. Aminov, W. Hu, A. V. Klieve, D. Ouwerkerk, M. A. Sundset, and Y. Kamagata. 2003. Ecology of uncultivated Oscillospira species in the rumen of cattle, sheep, and reindeer as assessed by microscopy and molecular approaches. Appl. Environ. Microbiol. 69:6808–6815. doi:10.1128/AEM.69.11.6808–6815.2003.

Mackie, R. I., C. S. McSweeney, and R. I. Aminov. 2001. Rumen. In: John Wiley & Sons, Ltd, editor. Encyclopedia of Life Sciences. John Wiley & Sons, Ltd, Chichester, UK. p. a0000404. Available from: http://doi.wiley.com/10.1038/npg.els.0000404

McDonald, P., J. F. D. Greenhalgh, C. A. Morgan, R. Edwards, and L. Sinclair. 2011. Animal nutrition. 7th ed. Pearson, New York, NY.

de Menezes, A. B., E. Lewis, M. O'Donovan, B. F. O'Neill, N. Clipson, and E. M. Doyle. 2011. Microbiome analysis of dairy cows fed pasture or total mixed ration diets. FEMS Microbiol. Ecol. 78:256–265. doi:10.1111/j.1574-6941.2011.01151.x.

Mullins, C. R., L. K. Mamedova, A. J. Carpenter, Y. Ying, M. S. Allen, I. Yoon, and B. J. Bradford. 2013. Analysis of rumen microbial populations in lactating dairy cattle fed diets varying in carbohydrate profiles and Saccharomyces cerevisiae fermentation product. J. Dairy Sci. 96:5872–5881. doi:10.3168/jds.2013-6775.

Münnich, M., A. Khol-Parisini, F. Klevenhusen, B. U. Metzler-Zebeli, and Q. Zebeli. 2018. Graded replacement of maize grain with molassed sugar beet pulp modulated ruminal microbial community and fermentation profile in vitro: Sugar beet pulp, rumen microbiota and fermentation in vitro. J. Sci. Food Agric. 98:991–997. doi:10.1002/jsfa.8547.

Myer, P. R., T. P. Smith, J. E. Wells, L. A. Kuehn, and H. C. Freetly. 2015. Rumen microbiome from steers differing in feed efficiency. PloS One. 10:e0129174. doi:doi.org/10.1371/journal.pone.0129174.

Newbrook, K., G. J. Staton, S. R. Clegg, R. J. Birtles, S. D. Carter, and N. J. Evans. 2017. Treponema ruminis sp. nov., a spirochaete isolated from the bovine rumen. Int. J. Syst. Evol. Microbiol. 67:1349–1354. doi:10.1099/ijsem.0.001812.

Niu, Q., P. Li, S. Hao, Y. Zhang, S. W. Kim, H. Li, X. Ma, S. Gao, L. He, W. Wu, X. Huang, J. Hua, B. Zhou, and R. Huang. 2015. Dynamic distribution of the gut microbiota and the relationship with apparent crude fiber digestibility and growth stages in pigs. Sci. Rep. 5:9938. doi:10.1038/srep09938.

Nordhoff, M., D. Taras, M. Macha, K. Tedin, H.-J. Busse, and L. H. Wieler. 2005. Treponema berlinense sp. nov. and Treponema porcinum sp. nov., novel spirochaetes isolated from porcine faeces. Int. J. Syst. Evol. Microbiol. 55:1675–1680. doi:10.1099/ijs.0.63388-0.

NRC. 2016. Nutrient requirements of beef cattle. 8th ed. National Academies Press, Washington, DC.

Petri, R. M., C. Mapiye, M. E. Dugan, and T. A. McAllister. 2014. Subcutaneous adipose fatty acid profiles and related rumen bacterial populations of steers fed red clover or grass hay diets containing flax or sunflower-seed. PloS One. 9:e104167. doi:doi.org/10.1371/journal.pone.0104167.

Petri, R. M., T. Schwaiger, G. B. Penner, K. A. Beauchemin, R. J. Forster, J. J. McKinnon, and T. A. McAllister. 2013. Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. PloS One. 8:e83424. doi:10.1371/journal.pone.0083424.

Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar, and R Core Team. 2020. nlme: Linear and nonlinear mixed effects models. R Foundation for Statistical Computing, Vienna, Austria. Available from: https://CRAN.R-project.org/package=nlme

Plaizier, J. C., S. Li, A. M. Danscher, H. Derakshani, P. H. Andersen, and E. Khafipour. 2017. Changes in microbiota in rumen digesta and feces due to a grain-based subacute ruminal acidosis (SARA) challenge. Microb. Ecol. 74:485–495. doi:10.1007/s00248-017-0940-z.

Plegge, S. D., L. L. Berger, and G. C. Fahey. 1985. Effect of roasting temperature on the proportion of soybean meal nitrogen escaping degradation in the rumen. J. Anim. Sci. 61:1211–1218. doi:10.2527/jas1985.6151211x.

Preston, T. R., and R. A. Leng. 1987. Matching ruminant production systems with available resources in the tropics and sub-tropics. Penambul Books, Armidale, N.S.W.

R Core Team. 2019. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Russell, J. B., J. D. O'Connor, D. G. Fox, P. J. Van Soest, and C. J. Sniffen. 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. J. Anim. Sci. 70:3551–3561. doi:10.2527/1992.70113551x.

Shabat, S. K. B., G. Sasson, A. Doron-Faigenboim, T. Durman, S. Yaacoby, M. E. Berg Miller, B. A. White, N. Shterzer, and I. Mizrahi. 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME J. 10:2958–2972. doi:10.1038/ismej.2016.62.

Shen, J., Z. Li, Z. Yu, and W. Zhu. 2020. Effects of dietary replacement of soybean meal with dried distillers grains with solubles on the microbiota occupying different ecological niches in the rumen of growing Hu lambs. J. Anim. Sci. Biotechnol. 11:93. doi:10.1186/s40104-020-00499-2.

Storm, E., E. R. Ørskov, and R. Smart. 1983. The nutritive value of rumen micro-organisms in ruminants: 2. The apparent digestibility and net utilization of microbial N for growing lambs. Br. J. Nutr. 50:471–478. doi:10.1079/bjn19830115.

Terrapon, N., V. Lombard, H. J. Gilbert, and B. Henrissat. 2015. Automatic prediction of polysaccharide utilization loci in Bacteroidetes species. Bioinformatics. 31:647–655. doi:10.1093/bioinformatics/btu716.

Wang, K., X. Nan, K. Chu, J. Tong, L. Yang, S. Zheng, G. Zhao, L. Jiang, and B. Xiong. 2018. Shifts of hydrogen metabolism from methanogenesis to propionate production in response to replacement of forage fiber with non-forage fiber sources in diets in vitro. Front. Microbiol. 9:2764. doi:10.3389/fmicb.2018.02764.

Wojciechowicz, M., and A. Ziołecki. 1979. Pectinolytic enzymes of large rumen treponemes. Appl. Environ. Microbiol. 37:136–142. doi:10.1128/AEM.37.1.136-142.1979.

Zhang, J., H. Shi, Y. Wang, S. Li, Z. Cao, S. Ji, Y. He, and H. Zhang. 2017. Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in Holstein heifers. Front. Microbiol. 8:2206. doi:10.3389/fmicb.2017.02206.

Zhang, R. Y., W. Jin, P. F. Feng, J. H. Liu, and S. Y. Mao. 2018. High-grain diet feeding altered the composition and functions of the rumen bacterial community and caused the damage to the laminar tissues of goats. Animal. 12:2511–2520. doi:10.1017/S175173111800040X.

Zhao, X. H., C. J. Liu, Y. Liu, C. Y. Li, and J. H. Yao. 2013. Effects of replacing dietary starch with neutral detergent-soluble fibre on ruminal fermentation, microbial synthesis and populations of ruminal cellulolytic bacteria using the rumen simulation technique (RUSITEC). J. Anim. Physiol. Anim. Nutr. 97:1161–1169. doi:10.1111/jpn.12025.

Zhu, Z., L. Kristensen, G. F. Difford, M. Poulsen, S. J. Noel, W. Abu Al-Soud, S. J. Sørensen, J. Lassen, P. Løvendahl, and O. Højberg. 2018. Changes in rumen bacterial and archaeal communities over the transition period in primiparous Holstein dairy cows. J. Dairy Sci. 101:9847–9862. doi:10.3168/jds.2017-14366.

TABLES

Table 1. Ingredients and nutrient inclusion for each treatment diet[1,2]

Ingredient, % of DM	SBM-TH	HSBM-TH	SBM-BP	HSBM-BP
Alfalfa hay	10.0	9.9	10.1	10.1
Corn	16.0	15.7	19.2	19.9
Barley	16.0	15.7	19.2	19.9
Soybean meal	13.9	0.00	16.1	0.00
Soyplus	0.00	14.4	0.00	18.1
Timothy hay	23.5	20.6	0.00	0.00
Beet pulp	0.00	0.00	42.0	41.0
Wheat middlings	20.0	23.0	2.20	0.50
Trace mineral salt	0.74	0.88	0.54	0.55
Vitamin premix	0.74	0.88	0.54	0.55
Nutrient, %[3]				
DM	96.2	96.2	96.2	96.2
CP	17.0	16.8	16.6	16.1
NDF	29.8	30.7	29.6	31.0
ADF	13.5	12.9	13.7	14.2
Lignin	2.11	1.80	1.96	2.19
Starch	27.3	26.6	27.9	24.9
Ash	4.87	4.22	4.79	4.96

[1]HSBM-TH = heat-treated soybean meal and timothy hay; SBM-TH = soybean meal and timothy hay; HSBM-BP = heat-treated soybean meal and beet pulp; SBM-BP = heat-treated soybean meal and beet pulp.
[2]Alfalfa hay, corn, barley, wheat middlings, trace mineral salt, and vitamin premix were incorporated into pellets along with either soybean meal or Soyplus to create the SBM and HSBM treatments, respectively.
[3]Nutrient percentages are expressed on a DM basis except for DM, which is expressed on an AF basis.

Table 2. LS means ± SE for percent relative abundances of bacterial taxa identified in the rumen fluid fraction as differentiated by diet along with *P* values for the effects of protein source, fiber source, and the interaction of protein and fiber source[1,2]

Taxonomic classification			Diet				*P* value		
Phylum	Family	Genus	SBM-TH	HSBM-TH	SBM-BP	HSBM-BP	Protein	Fiber	Protein × Fiber
Euryarchaeota (domain Archaea)			0.0113 ± 0.061	0.232 ± 0.061	0.157 ± 0.064	0.129 ± 0.064	0.39	0.45	0.24
Euryarchaeota (domain Archaea)	*Methanomassiliicoccaceae*		0.0950 ± 0.058	0.230 ± 0.058	0.121 ± 0.062	0.118 ± 0.061	0.23	0.32	0.26
Bacteroidetes			78.5 ± 4.5	73.1 ± 4.4	77.6 ± 4.7	78.6 ± 4.7	0.61	0.61	0.47
Bacteroidetes	BS11		1.20 ± 0.74	0.0354 ± 0.74	0.391 ± 0.77	0.790 ± 0.77	0.46	0.95	0.19
Bacteroidetes	p-2534-18B5		1.76 ± 3.0	1.57 ± 3.0	7.15 ± 3.2	3.47 ± 3.1	0.57	0.27	0.57
Bacteroidetes	*Paraprevotellaceae*		2.55 ± 1.5	2.20 ± 1.5	1.78 ± 1.6	4.60 ± 1.5	0.38	0.74	0.28
Bacteroidetes	*Paraprevotellaceae*	CF231	1.93 ± 1.0	1.27 ± 1.0	1.17 ± 1.1	0.268 ± 1.1	0.42	0.25	0.89
Bacteroidetes	*Paraprevotellaceae*	YRC22	0.416 ± 0.31	0.424 ± 0.31	0.112 ± 0.32	2.04 ± 0.32	**0.0072**	**0.052**	**0.0056**
Bacteroidetes	*Porphyromonadaceae*		0.155 ± 0.14	0.202 ± 0.13	0.0291 ± 0.14	0.00406 ± 0.14	0.89	0.17	0.80
Bacteroidetes	*Prevotellaceae*		58.3 ± 6.6	63.7 ± 6.6	64.3 ± 7.0	64.9 ± 7.0	0.66	0.48	0.71
Bacteroidetes	*Prevotellaceae*	*Prevotella*	58.0 ± 6.6	63.4 ± 6.6	64.0 ± 7.0	64.7 ± 7.0	0.65	0.47	0.72
Bacteroidetes	RF16		0.180 ± 0.19	0.299 ± 0.19	0.186 ± 0.20	0.268 ± 0.20	0.56	0.78	0.92
Bacteroidetes	S24-7		1.07 ± 0.39	0.758 ± 0.39	0.585 ± 0.41	0.325 ± 0.41	0.49	0.23	0.95
Cyanobacteria			0.0804 ± 1.2	0.0968 ± 1.1	2.17 ± 1.2	1.84 ± 1.2	0.91	0.11	0.88
Fibrobacteres			3.58 ± 3.6	8.45 ± 3.6	3.48 ± 3.8	6.55 ± 3.8	0.30	0.75	0.81
Fibrobacteres	*Fibrobacteraceae*		3.58 ± 3.6	8.45 ± 3.6	3.48 ± 3.8	6.55 ± 3.8	0.30	0.75	0.81
Fibrobacteres	*Fibrobacteraceae*	*Fibrobacter*	3.58 ± 3.6	8.45 ± 3.6	3.48 ± 3.8	6.55 ± 3.8	0.30	0.75	0.81
Firmicutes			8.20 ± 1.8	6.00 ± 1.8	4.85 ± 1.9	6.75 ± 1.86	0.93	0.31	0.23
Firmicutes	*Erysipelotrichaceae*		3.98 ± 1.5	1.11 ± 1.5	1.27 ± 1.6	3.04 ± 1.6	0.68	0.60	0.11
Firmicutes	*Erysipelotrichaceae*	*Asteroleplasma*	2.56 ± 1.6	0.151 ± 1.6	0.565 ± 1.6	1.31 ± 1.6	0.52	0.67	0.25
Firmicutes	*Erysipelotrichaceae*	RFN20	1.34 ± 0.48	0.655 ± 0.48	0.562 ± 0.50	0.268 ± 0.50	0.34	0.15	0.69
Firmicutes	*Erysipelotrichaceae*	*Sharpea*	0.0696 ± 0.62	0.285 ± 0.61	0.153 ± 0.65	1.44 ± 0.65	0.24	0.38	0.39
Firmicutes	*Lachnospiraceae*		1.09 ± 0.45	0.547 ± 0.44	1.17 ± 0.47	1.53 ± 0.47	0.81	0.27	0.33
Firmicutes	*Lachnospiraceae*	*Butyrivibrio*	0.266 ± 0.36	0.161 ± 0.36	0.469 ± 0.38	0.967 ± 0.37	0.59	0.22	0.41
Firmicutes	*Lachnospiraceae*	*Lachnospira*	0.0701 ± 0.11	0.0253 ± 0.11	0.262 ± 0.12	0.318 ± 0.12	0.97	**0.022**	0.63
Firmicutes	*Lachnospiraceae*	*Roseburia*	0.571 ± 0.21	0.166 ± 0.21	0.0379 ± 0.22	0.0210 ± 0.22	0.29	0.15	0.37
Firmicutes	*Ruminococcaceae*		1.73 ± 0.64	0.931 ± 0.64	0.595 ± 0.68	0.263 ± 0.67	0.40	0.14	0.72
Firmicutes	*Ruminococcaceae*	*Oscillospira*	0.274 ± 0.095	0.239 ± 0.095	0.0100 ± 0.10	0.0277 ± 0.10	0.87	**0.015**	0.76
Firmicutes	*Ruminococcaceae*	*Ruminococcus*	1.37 ± 0.60	0.575 ± 0.60	0.547 ± 0.63	0.0985 ± 0.63	0.33	0.23	0.78
Firmicutes	*Veillonellaceae*		0.704 ± 0.68	2.85 ± 0.68	1.52 ± 0.72	0.894 ± 0.71	0.26	0.48	**0.058**
Firmicutes	*Veillonellaceae*	*Acidaminococcus*	0.0524 ± 0.060	0.205 ± 0.060	0.0349 ± 0.064	0.0588 ± 0.063	0.15	0.24	0.31
Firmicutes	*Veillonellaceae*	*Megasphaera*	0.0826 ± 0.062	0.0644 ± 0.062	0.0324 ± 0.066	0.226 ± 0.066	0.22	0.27	0.10

Table 2 cont'd.

Firmicutes	*Veillonellaceae*	*Selenomonas*	0.368 ± 0.70	2.10 ± 0.70	1.14 ± 0.74	0.263 ± 0.74	0.52	0.51	**0.082**
Firmicutes	*Veillonellaceae*	*Succiniclasticum*	0.123 ± 0.10	0.407 ± 0.10	0.173 ± 0.11	0.137 ± 0.11	0.17	0.23	0.11
Proteobacteria			5.65 ± 2.1	7.05 ± 2.1	3.33 ± 2.2	2.55 ± 2.2	0.93	0.23	0.62
Proteobacteria	*Succinivibrionaceae*		5.64 ± 2.1	7.01 ± 2.1	3.29 ± 2.2	2.5 ± 2.2	0.93	0.23	0.63
Proteobacteria	*Succinivibrionaceae*	*Ruminobacter*	0.00372 ± 0.14	0.0306 ± 0.14	0.188 ± 0.15	0.336 ± 0.15	0.53	0.13	0.68
Proteobacteria	*Succinivibrionaceae*	*Succinivibrio*	3.73 ± 1.2	2.57 ± 1.2	0.748 ± 1.2	1.06 ± 1.2	0.65	0.16	0.54
Spirochaetes			2.52 ± 1.4	1.91 ± 1.4	7.84 ± 1.5	2.71 ± 1.5	**0.071**	**0.060**	0.13
Spirochaetes	*Sphaerochaetaceae*		0.892 ± 1.0	1.27 ± 1.0	3.08 ± 1.1	0.593 ± 1.1	0.36	0.52	0.19
Spirochaetes	*Sphaerochaetaceae*	*Sphaerochaeta*	0.892 ± 1.0	1.27 ± 1.0	3.08 ± 1.1	0.593 ± 1.1	0.36	0.52	0.19
Spirochaetes	*Spirochaetaceae*		1.63 ± 1.2	0.644 ± 1.2	4.73 ± 1.3	2.09 ± 1.3	0.15	**0.080**	0.48
Spirochaetes	*Spirochaetaceae*	*Treponema*	1.62 ± 1.2	0.644 ± 1.2	4.73 ± 1.3	2.09 ± 1.3	0.15	**0.080**	0.47
Tenericutes			0.611 ± 1.0	2.50 ± 1.0	0.346 ± 1.1	0.625 ± 1.07	0.28	0.36	0.43
Tenericutes	*Anaeroplasmataceae*		0.344 ± 0.21	0.444 ± 0.21	0.311 ± 0.22	0.403 ± 0.22	0.62	0.85	0.98
Tenericutes	*Anaeroplasmataceae*	*Anaeroplasma*	0.204 ± 0.15	0.436 ± 0.14	0.0408 ± 0.15	0.0151 ± 0.15	0.47	**0.057**	0.39
Verrucomicrobia			0.406 ± 0.14	0.332 ± 0.14	0.110 ± 0.14	0.122 ± 0.14	0.84	**0.037**	0.74
Verrucomicrobia	RFP12		0.396 ± 0.13	0.331 ± 0.13	0.108 ± 0.14	0.121 ± 0.14	0.88	**0.037**	0.76

[1]Taxa with relative abundances >0.1% for the 4 diet groups.

[2]HSBM-TH = heat-treated soybean meal and timothy hay; SBM-TH = soybean meal and timothy hay; HSBM-BP = heat-treated soybean meal and beet pulp; SBM-BP = heat-treated soybean meal and beet pulp.

Table 3. LS means ± SE for percent relative abundances of bacterial taxa identified in the rumen solid fraction as differentiated by diet along with *P* values for the effects of protein source, fiber source, and the interaction of protein and fiber source[1,2]

Taxonomic classification			Diet				*P* value		
Phylum	Family	Genus	SBM-TH	HSBM-TH	SBM-BP	HSBM-BP	Protein	Fiber	Protein × Fiber
Euryarchaeota (domain Archaea)			0.447 ± 0.23	0.670 ± 0.22	0.408 ± 0.23	0.155 ± 0.23	0.91	0.15	0.30
Euryarchaeota (domain Archaea)	*Methanomassiliicoccaceae*		0.436 ± 0.19	0.666 ± 0.18	0.165 ± 0.19	0.129 ± 0.19	0.46	**0.021**	0.48
Euryarchaeota (domain Archaea)	*Methanomassiliicoccaceae*	vadinCA11	0.172 ± 0.071	0.160 ± 0.067	0.0862 ± 0.071	0.0730 ± 0.071	0.95	0.17	0.99
Actinobacteria			0.334 ± 0.13	0.176 ± 0.12	0.0471 ± 0.12	0.0334 ± 0.13	0.43	0.12	0.55
Actinobacteria	*Coriobacteriaceae*		0.162 ± 0.067	0.144 ± 0.064	0.0382 ± 0.067	0.0310 ± 0.067	0.77	**0.089**	0.93
Actinobacteria	*Coriobacteriaceae*	*Olsenella*	0.139 ± 0.061	0.140 ± 0.058	0.0339 ± 0.061	0.0268 ± 0.061	0.90	**0.085**	0.94
Bacteroidetes			74.5 ± 5.7	73.3 ± 5.4	52.4 ± 5.7	65.9 ± 5.6	0.26	**0.014**	0.17
Bacteroidetes	BS11		0.0634 ± 0.33	0.0186 ± 0.31	0.0847 ± 0.33	0.657 ± 0.33	0.43	0.33	0.34
Bacteroidetes	*Bacteroidaceae*		0.128 ± 0.038	0.0147 ± 0.036	0.00685 ± 0.038	0.00233 ± 0.037	0.13	**0.080**	0.16
Bacteroidetes	*Bacteroidaceae*	BF311	0.122 ± 0.038	0.0103 ± 0.036	0.00369 ± 0.038	0.000362 ± 0.038	0.15	**0.092**	0.16
Bacteroidetes	p-2534-18B5		0.0356 ± 1.5	1.12 ± 1.4	1.34 ± 1.5	2.78 ± 1.5	0.37	0.38	0.92
Bacteroidetes	*Paraprevotellaceae*		1.77 ± 0.92	2.27 ± 0.87	1.03 ± 0.92	3.16 ± 0.92	0.15	0.93	0.37
Bacteroidetes	*Paraprevotellaceae*	CF231	0.448 ± 0.26	0.419 ± 0.25	0.403 ± 0.26	0.284 ± 0.26	0.81	0.54	0.85
Bacteroidetes	*Paraprevotellaceae*	YRC22	0.572 ± 0.30	1.12 ± 0.29	0.282 ± 0.30	1.16 ± 0.30	**0.023**	0.71	0.57
Bacteroidetes	*Porphyromonadaceae*		0.167 ± 0.15	0.134 ± 0.15	0.130 ± 0.15	0.122 ± 0.15	0.40	0.26	0.61
Bacteroidetes	*Prevotellaceae*		60.0 ± 7.0	59.9 ± 6.7	42.6 ± 7.0	52.9 ± 7.0	0.43	**0.089**	0.42
Bacteroidetes	*Prevotellaceae*	*Prevotella*	58.9 ± 7.0	59.0 ± 6.7	42.1 ± 7.0	52.6 ± 7.0	0.42	0.11	0.42
Bacteroidetes	RF16		0.123 ± 0.11	0.128 ± 0.11	0.0395 ± 0.11	0.220 ± 0.11	0.37	0.89	0.44
Bacteroidetes	S24-7		4.20 ± 1.0	4.03 ± 0.97	2.04 ± 1.0	0.396 ± 1.0	0.32	**0.0050**	0.42
Cyanobacteria			0.199 ± 0.75	0.0954 ± 0.72	1.06 ± 0.76	1.36 ± 0.75	0.96	0.16	0.79
Elusimicrobia			0.0437 ± 0.13	0.221 ± 0.12	0.0297 ± 0.13	0.00133 ± 0.13	0.54	0.37	0.43
Fibrobacteres			4.60 ± 3.5	8.41 ± 3.3	14.1 ± 3.5	7.23 ± 3.5	0.71	0.24	0.14
Fibrobacteres	*Fibrobacteraceae*		4.60 ± 3.5	8.41 ± 3.3	14.1 ± 3.5	7.23 ± 3.5	0.71	0.24	0.14
Fibrobacteres	*Fibrobacteraceae*	*Fibrobacter*	4.60 ± 3.5	8.41 ± 3.3	14.1 ± 3.5	7.23 ± 3.5	0.71	0.24	0.14
Firmicutes			10.3 ± 1.8	7.93 ± 1.7	4.67 ± 1.8	4.65 ± 1.8	0.44	**0.0054**	0.43
Firmicutes	*Clostridiaceae*		0.341 ± 0.11	0.268 ± 0.11	0.0380 ± 0.11	0.0293 ± 0.11	0.68	**0.0094**	0.74
Firmicutes	*Clostridiaceae*	*Clostridium*	0.341 ± 0.11	0.268 ± 0.11	0.0380 ± 0.11	0.0293 ± 0.11	0.68	**0.0094**	0.74
Firmicutes	*Erysipelotrichaceae*		3.13 ± 1.1	2.83 ± 1.0	1.48 ± 1.1	1.04 ± 1.1	0.71	**0.088**	0.94

Table 3 cont'd.

Firmicutes	*Erysipelotrichaceae*	*Asteroleplasma*	0.808 ± 0.47	0.0383 ± 0.44	0.287 ± 0.47	0.377 ± 0.46	0.43	0.74	0.29
Firmicutes	*Erysipelotrichaceae*	RFN20	0.730 ± 0.30	0.536 ± 0.28	0.396 ± 0.30	0.134 ± 0.30	0.52	0.12	0.90
Firmicutes	*Erysipelotrichaceae*	*Sharpea*	1.56 ± 0.87	2.16 ± 0.83	0.766 ± 0.87	0.470 ± 0.868	0.90	0.19	0.58
Firmicutes	*Lachnospiraceae*		2.37 ± 0.46	1.59 ± 0.43	1.66 ± 0.46	1.62 ± 0.45	0.34	0.43	0.39
Firmicutes	*Lachnospiraceae*	*Butyrivibrio*	1.63 ± 0.34	0.927 ± 0.32	0.797 ± 0.34	0.850 ± 0.34	0.32	0.19	0.26
Firmicutes	*Lachnospiraceae*	*Coprococcus*	0.0478 ± 0.030	0.107 ± 0.028	0.00635 ± 0.030	0.0447 ± 0.030	0.12	0.12	0.73
Firmicutes	*Lachnospiraceae*	*Lachnospira*	0.0128 ± 0.054	0.0035 ± 0.051	0.163 ± 0.054	0.148 ± 0.054	0.72	**0.0049**	0.99
Firmicutes	*Lactobacillaceae*		0.221 ± 0.10	0.100 ± 0.099	0.0200 ± 0.10	0.00124 ± 0.10	0.51	0.16	0.62
Firmicutes	*Lactobacillaceae*	*Lactobacillus*	0.221 ± 0.10	0.100 ± 0.099	0.0200 ± 0.10	0.00124 ± 0.10	0.51	0.16	0.62
Firmicutes	*Ruminococcaceae*		0.985 ± 0.37	1.15 ± 0.35	0.297 ± 0.37	0.443 ± 0.37	0.64	**0.064**	0.98
Firmicutes	*Ruminococcaceae*	*Ruminococcus*	0.880 ± 0.35	1.04 ± 0.33	0.176 ± 0.35	0.300 ± 0.35	0.65	**0.048**	0.96
Firmicutes	*Veillonellaceae*		1.45 ± 0.27	1.36 ± 0.25	0.768 ± 0.27	0.644 ± 0.27	0.72	**0.016**	0.95
Firmicutes	*Veillonellaceae*	*Acidaminococcus*	0.314 ± 0.074	0.236 ± 0.070	0.137 ± 0.074	0.0965 ± 0.074	0.33	**0.041**	0.78
Firmicutes	*Veillonellaceae*	*Megasphaera*	0.242 ± 0.12	0.0261 ± 0.11	0.0433 ± 0.12	0.0136 ± 0.12	0.27	0.43	0.43
Firmicutes	*Veillonellaceae*	*Mitsuokella*	0.158 ± 0.050	0.118 ± 0.048	0.0835 ± 0.050	0.145 ± 0.050	0.96	0.85	0.32
Firmicutes	*Veillonellaceae*	*Selenomonas*	0.200 ± 0.099	0.313 ± 0.094	0.221 ± 0.099	0.0727 ± 0.099	0.94	0.24	0.19
Firmicutes	*Veillonellaceae*	*Succiniclasticum*	0.486 ± 0.17	0.637 ± 0.16	0.149 ± 0.17	0.163 ± 0.17	0.51	**0.0099**	0.65
Planctomycetes			0.232 ± 0.12	0.00466 ± 0.11	0.0475 ± 0.12	0.00170 ± 0.12	0.26	0.40	0.44
Planctomycetes	*Pirellulaceae*		0.232 ± 0.12	0.00466 ± 0.11	0.0475 ± 0.12	0.00170 ± 0.12	0.26	0.40	0.44
Proteobacteria			1.63 ± 0.63	1.44 ± 0.59	0.862 ± 0.63	1.23 ± 0.62	0.94	0.51	0.66
Proteobacteria	*Succinivibrionaceae*		1.55 ± 0.62	1.36 ± 0.59	0.827 ± 0.62	1.20 ± 0.62	0.93	0.56	0.65
Proteobacteria	*Succinivibrionaceae*	*Ruminobacter*	0.220 ± 0.095	0.0332 ± 0.090	0.0569 ± 0.95	0.0106 ± 0.095	0.27	0.26	0.46
Proteobacteria	*Succinivibrionaceae*	*Succinivibrio*	1.18 ± 0.49	0.327 ± 0.46	0.231 ± 0.49	0.930 ± 0.49	0.74	0.94	0.12
Spirochaetes			6.69 ± 6.1	5.62 ± 5.8	25.6 ± 6.1	18.7 ± 6.0	0.47	**0.013**	0.61
Spirochaetes	*Sphaerochaetaceae*		0.212 ± 0.20	0.196 ± 0.19	0.571 ± 0.20	0.239 ± 0.20	0.30	0.27	0.34
Spirochaetes	*Sphaerochaetaceae*	*Sphaerochaeta*	0.212 ± 0.20	0.196 ± 0.19	0.571 ± 0.20	0.239 ± 0.20	0.30	0.27	0.34
Spirochaetes	*Spirochaetaceae*		6.50 ± 6.1	5.42 ± 5.8	25.1 ± 6.1	18.5 ± 6.1	0.50	**0.015**	0.63
Spirochaetes	*Spirochaetaceae*	*Treponema*	6.48 ± 6.1	5.42 ± 5.8	25.1 ± 6.1	18.5 ± 6.1	0.50	**0.015**	0.63
SR1			0.421 ± 0.50	0.420 ± 0.50	0.422 ± 0.50	0.426 ± 0.50	0.64	0.16	0.38
Tenericutes			0.749 ± 0.43	1.51 ± 0.40	0.450 ± 0.43	0.244 ± 0.43	0.49	**0.083**	0.26
Tenericutes	*Anaeroplasmataceae*		0.442 ± 0.19	0.645 ± 0.18	0.166 ± 0.19	0.121 ± 0.19	0.56	**0.020**	0.47
Tenericutes	*Anaeroplasmataceae*	*Anaeroplasma*	0.401 ± 0.18	0.644 ± 0.18	0.0866 ± 0.18	0.0159 ± 0.18	0.53	**0.0081**	0.37

[1]Taxa with relative abundances >0.1% for the 4 diet groups.

[2]HSBM-TH = heat-treated soybean meal and timothy hay; SBM-TH = soybean meal and timothy hay; HSBM-BP = heat-treated soybean meal and beet pulp; SBM-BP = heat-treated soybean meal and beet pulp.

Table 4. LS means ± SE for alpha diversity metrics of the bacterial community identified in the rumen fluid and solid fractions as differentiated by diet along with *P* values for the effects of protein source, fiber source, and the interaction of protein and fiber source[1]

	Diet				*P* value		
Measurement	SBM-TH	HSBM-TH	SBM-BP	HSBM-BP	Protein	Fiber	Protein × Fiber
Fluid fraction							
Observed OTUs	167 ± 23	172 ± 23	152 ± 24	153 ± 24	0.84	0.31	0.92
Shannon diversity	3.73 ± 0.30	4.11 ± 0.30	3.62 ± 0.31	3.7 ± 0.31	0.27	0.19	0.52
Solid fraction							
Observed OTUs	229 ± 30	207 ± 28	176 ± 30	154 ± 29	0.53	**0.051**	0.99
Shannon diversity	4.62 ± 0.30	4.78 ± 0.29	3.87 ± 0.30	3.85 ± 0.30	0.69	**0.0027**	0.75

[1]HSBM-TH = heat-treated soybean meal and timothy hay; SBM-TH = soybean meal and timothy hay; HSBM-BP = heat-treated soybean meal and beet pulp; SBM-BP = heat-treated soybean meal and beet pulp; OTUs = operational taxonomic units.

FIGURES

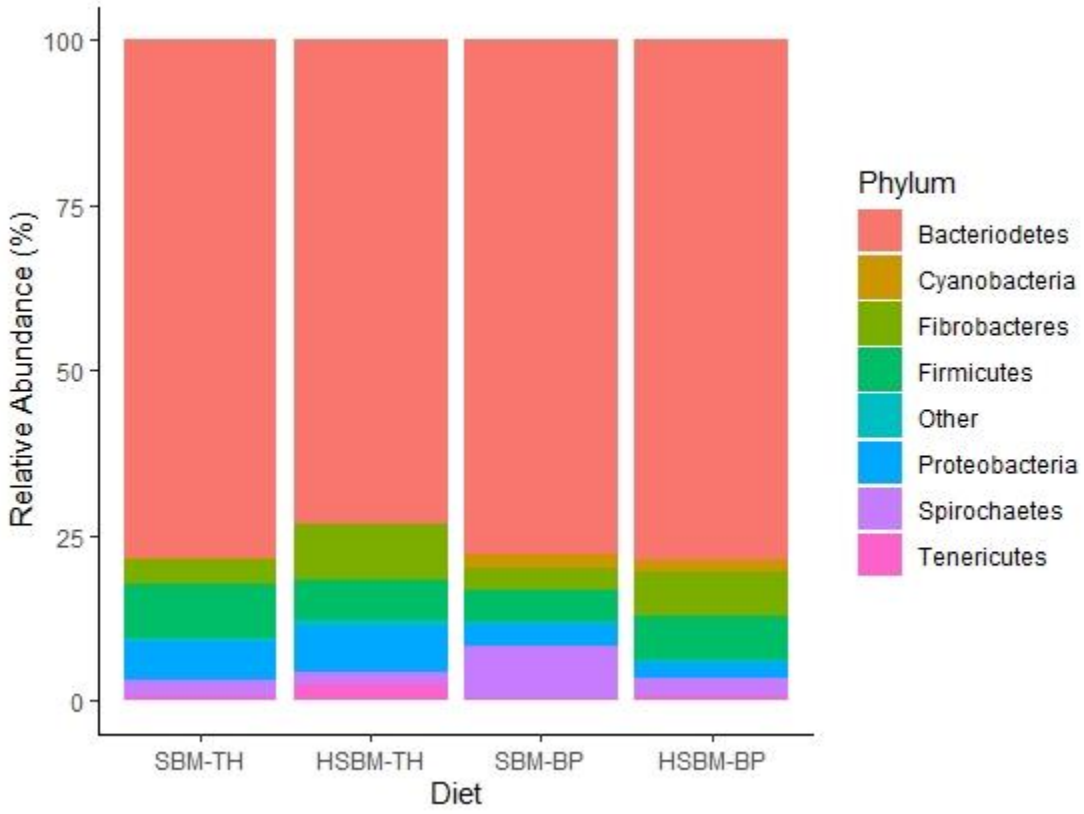

Figure 1a. Phylum-level composition of bacteria identified in the rumen fluid fraction as differentiated by diet. The category "Other" is comprised of taxa with relative abundances < 1.0% for all diets.

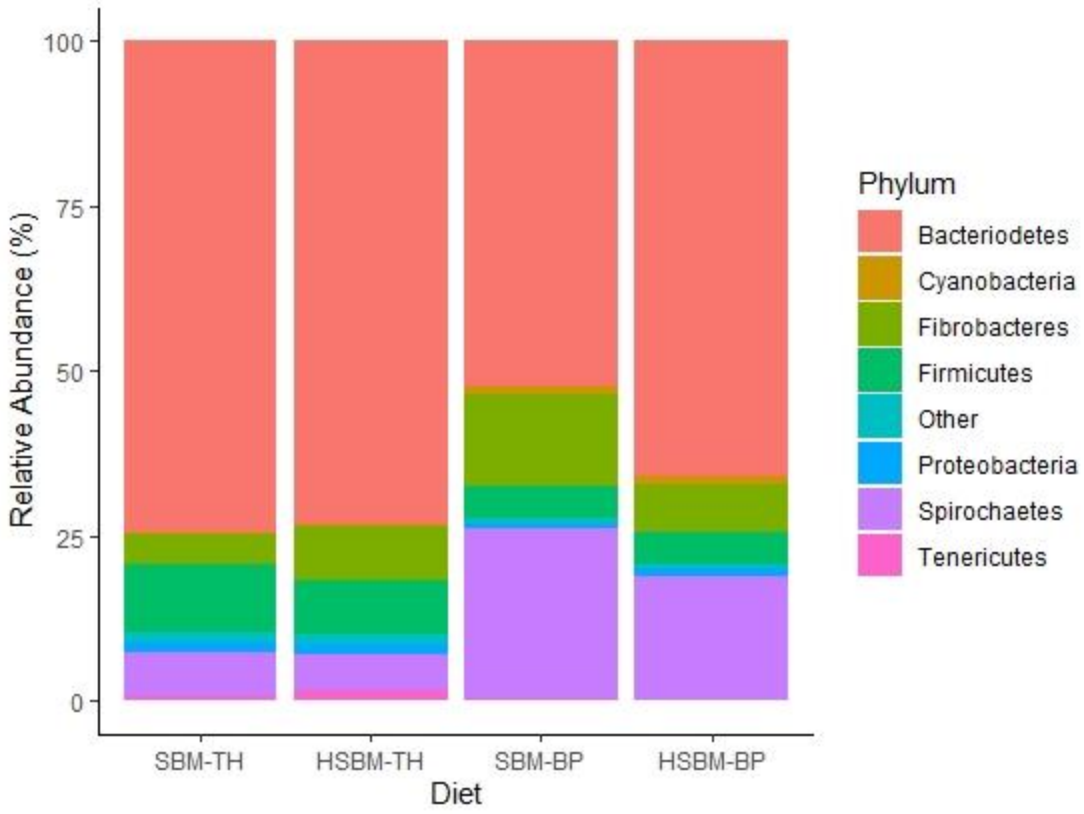

Figure 1b. Phylum-level composition of bacteria identified in the rumen solid fraction as differentiated by diet. The category "Other" is comprised of taxa with relative abundances < 1.0% for all diets.

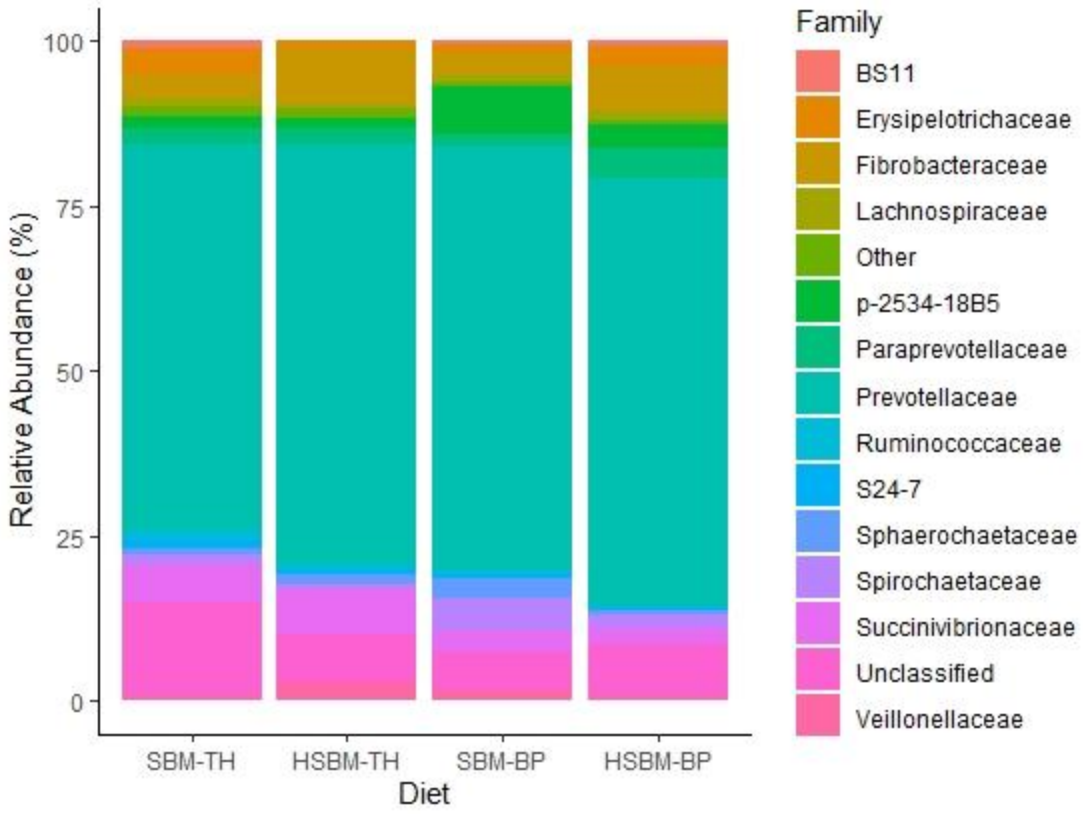

Figure 2a. Family-level composition of bacteria identified in the rumen fluid fraction as differentiated by diet. The category "Other" is comprised of taxa with relative abundances < 1.0% for all diets.

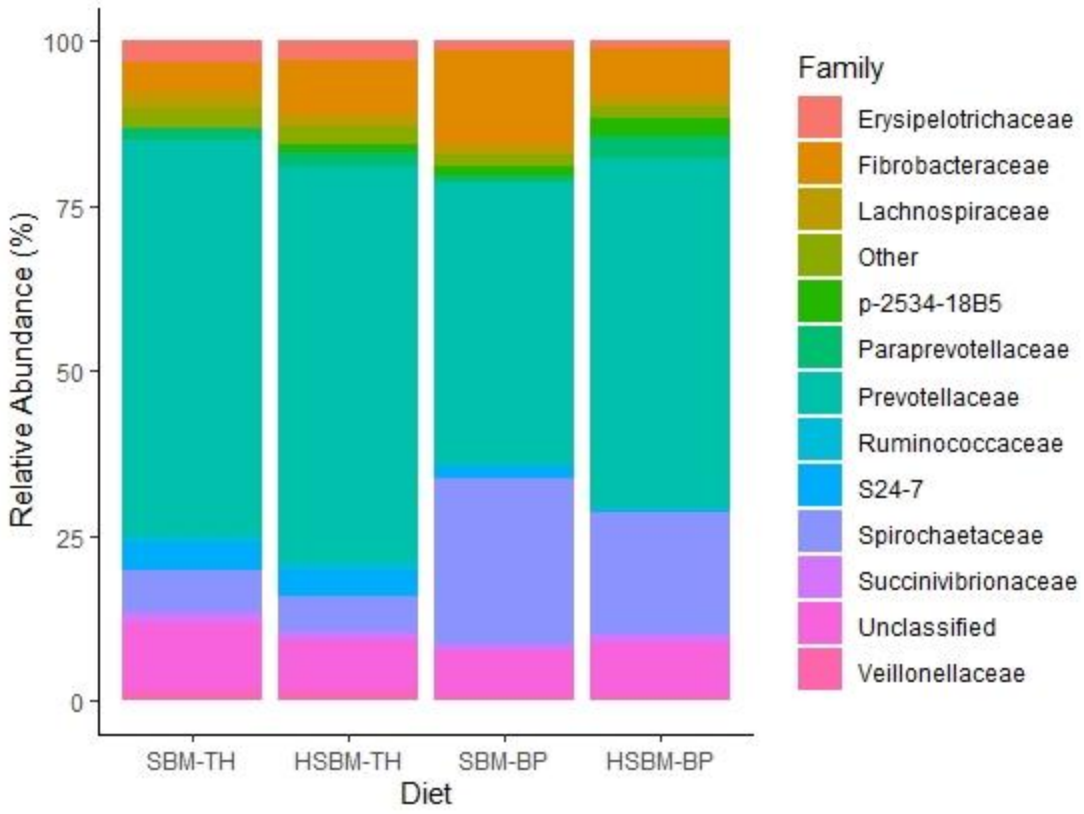

Figure 2b. Family-level composition of bacteria identified in the rumen solid fraction as differentiated by diet. The category "Other" is comprised of taxa with relative abundances < 1.0% for all diets.

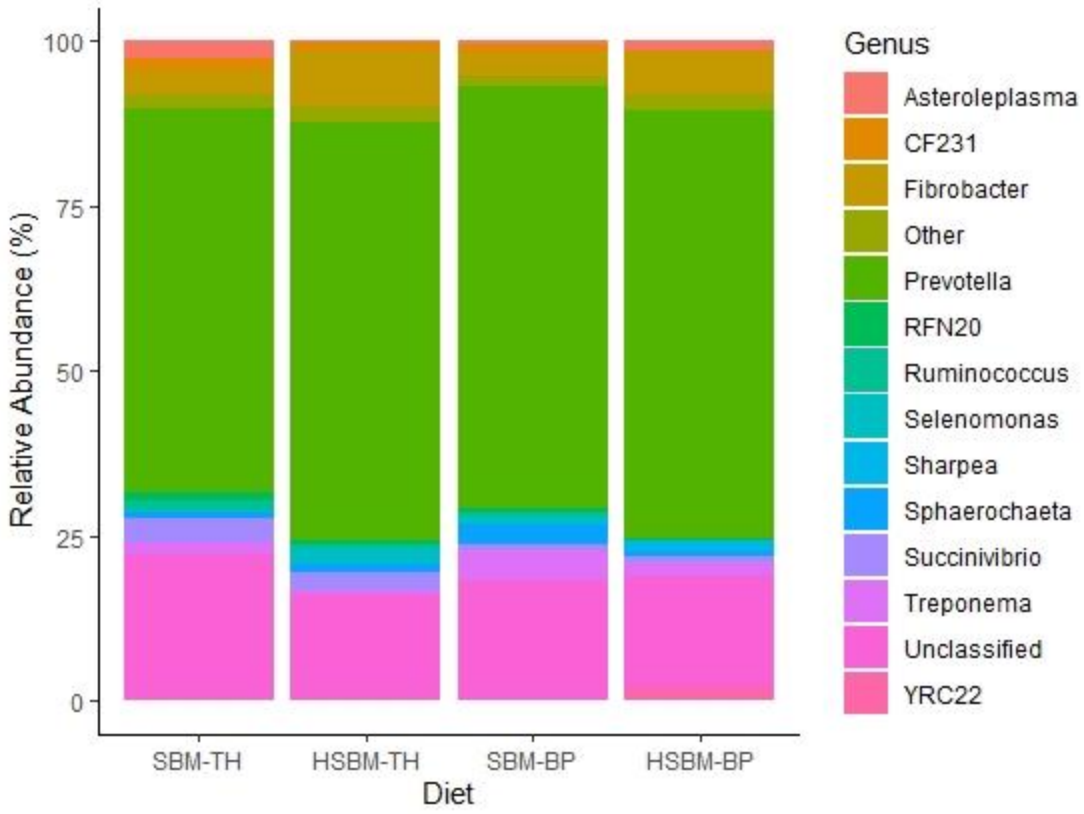

Figure 3a. Genus-level composition of bacteria identified in the rumen fluid fraction as differentiated by diet. The category "Other" is comprised of taxa with relative abundances < 1.0% for all diets.

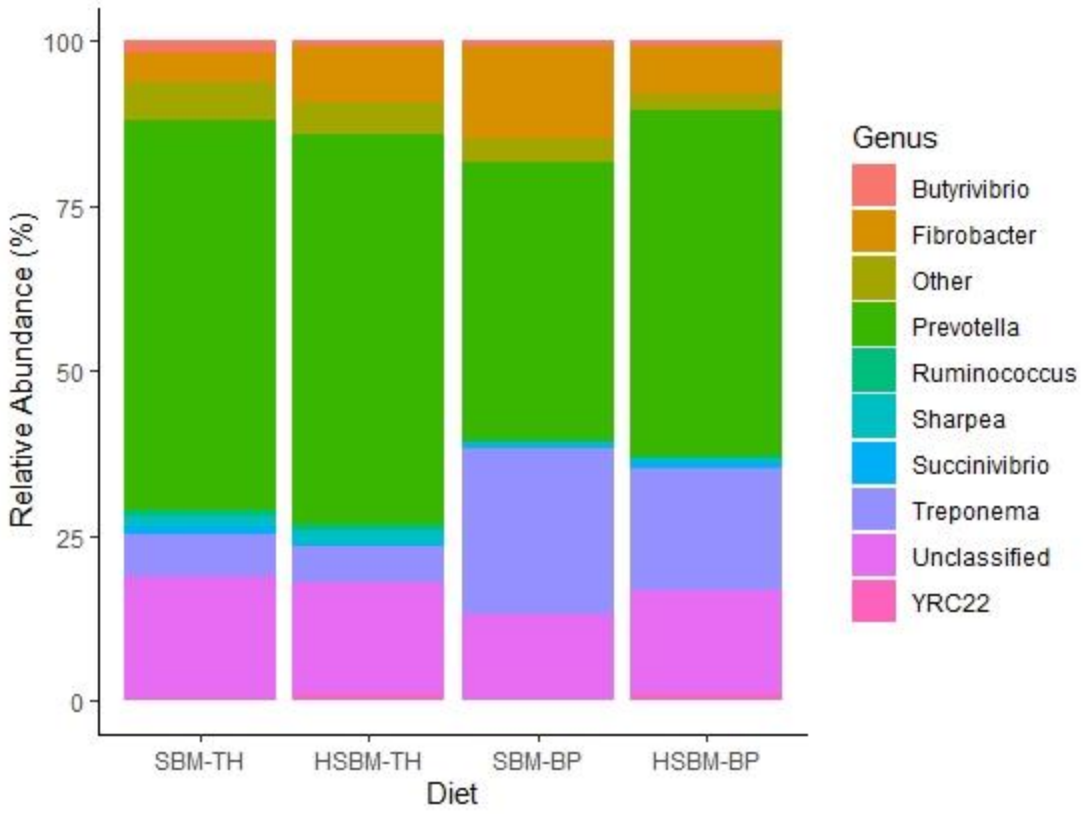

Figure 3b. Genus-level composition of bacteria identified in the rumen solid fraction as differentiated by diet. The category "Other" is comprised of taxa with relative abundances < 1.0% for all diets.

GENERAL CONCLUSION

Volatile fatty acids represent a critically important energy source that ruminant animals rely on for the support of body maintenance and productive activities such as growth, reproduction, and milk production. These productive outputs contribute directly to the security of the human food supply and represent valuable sources of essential nutrients in the form of meat and dairy products. The supply of VFA to the animal is known to be impacted by numerous nutritional, biochemical, and microbial factors, but how these factors interconnect and influence rumen VFA dynamics has not been well characterized or understood. The central aim of this work was therefore to investigate how fermentation dynamics, rumen environmental characteristics, and the rumen microbiome behave in response to variations in fermentative substrate supply.

Chapter 3 described the evaluation of starch degradation rates obtained by using a novel *in vitro* approach or a traditional *in situ* approach. The experimental objectives were to 1) assess starch degradation of a variety of livestock feedstuffs using an *in vitro* technique based on an official method of starch analysis and 2) assess how the enzymatic *in vitro* starch degradation assay compared with the *in situ* technique in terms of its ability to rank feeds based on their starch degradation rates. A relationship between the degradation rates estimated by these procedures was observed, but only when feed nutrient content was accounted for. Even though this *in vitro* method may not be able to reflect *in vivo* ruminal starch degradation rates, it holds potential as a laboratory technique for ranking feeds based on their relative differences in starch degradability. Starch is an important substrate for fermentation and such a laboratory tool to evaluate livestock feeds based on their estimated relative starch degradation characteristics will

be valuable for ration formulation efforts. Future work should focus on determining the repeatability of this assay and the extent of its usefulness.

In Chapter 4, we presented an investigation into ruminal fermentation and epithelial gene expression responses to diets containing ingredients designed to differ in rumen availabilities of key fermentative substrates. The objective was to characterize how variations in protein and fiber sources affect dry matter intake, fermentation parameters, rumen pH, rumen fluid dynamics, and epithelial gene expression levels. Timothy hay and beet pulp were utilized as the fiber sources while soybean meal and heat-treated soybean meal represented the protein sources. This investigation discovered numerous shifts in VFA fermentation dynamics in response to changes in both nutrient sources, predominantly involving interconversions between different VFA. It was also determined that intake, rumen environmental parameters, and epithelial gene expression levels were resistant to influence by variations in fiber or protein source. This is an important finding as it suggests we can act flexibly in formulating rations with these ingredients to optimize fermentation without eliciting detrimental effects such as depressed rumen pH or compromised epithelial function. To the best of our knowledge, this is the first report to concurrently evaluate the effects of varying sources of nutrients on VFA dynamics, rumen environmental variables, and rumen epithelial gene expression. This work also appears to be the first of its kind to characterize dietary substrate influences of intraruminal carbon exchanges involving the minor VFA. Further study is required to determine how these dietary effects may impact postabsorptive metabolism and animal performance.

Chapter 5 presented a meta-analysis of the available literature that was performed to ascertain if the rumen microbiome could be used to help explain variation in beef and dairy performance measures. The overall objective was to determine whether the microbiome could be

an accurate predictor of animal productivity and compare the strength of its predictions with predictions using diet variables. The microbial variables utilized included three prominent rumen bacterial phyla, species richness, and species diversity, while the diet variables included nutrient chemical composition and forage percentage. Assessments of model quality indicated that performance predictions based on the microbiome may be similar in accuracy to those determined using diet variables and that microbial variables may be used to enhance predictions in combination with diet. Diet and the microbiome may therefore exert effects independent of each other on various aspects of animal performance. Future work should attempt to model animal performance using more specific microbial variables, such as abundance at the genus or species level, and metatranscriptomics variables.

Chapter 6, the final chapter, explored changes in the rumen microbiome in response to ingredients differing in ruminally degradable protein and fiber supplies, the same treatments described in Chapter 3. The objective was to investigate the effects of different fiber and protein sources on rumen microbial population abundances at the phylum, family, and genus levels, as well as estimations of community richness and diversity. Substantial population shifts occurred in response to the differing fiber sources, with decreases in numerous fibrolytic populations and overall richness and diversity associated with beet pulp treatment. Optimal fiber substrate type varies between different bacterial populations involved in its breakdown and fibrolytic species may not be as adequately supported by non-forage fiber sources. Future ruminant nutrition work must therefore begin to move away from broad classification of nutrients and towards the characterization of specific substrate supplies. The decreases in species richness and diversity observed in response to beet pulp treatment are indicators that overall microbiome efficiency was enhanced under that treatment because previous investigations, including the meta-analysis

presented in Chapter 5, have established links between decreased richness and diversity and increased animal efficiency. Additional study is necessary to determine if the differing nutrient sources utilized in this investigation and their subsequent influence on the rumen microbiome translate into relevant alterations in animal performance.

As the demand for meat and dairy products continues to increase with the growth of the global population, the ruminant livestock industries must continue to devise and improve strategies aimed at increasing animal productivity under the prospect of restricted resources and climate change concerns. Gaining an improved view of how the rumen works puts us in a better position to manipulate fermentative outcomes via the diet to increase animal efficiency. The simplistic, reductionist approach often taken in ruminant nutrition research whereby only a few variables are considered at a time is inadequate for making meaningful progress in gaining a more comprehensive understanding of the exceptionally complex process of rumen fermentation. The work presented here has succeeded in concurrently analyzing a large number of variables impacting the rumen system and represents the approach necessary for tackling poorly understood aspects of the fermentative process. Overall, this body of work helps to advance our understanding of the complex factors that drive rumen fermentation by taking a holistic approach in quantifying responses of VFA dynamics, microbial populations, rumen environmental variables, and rumen epithelial characteristics to variations in the provided sources of fermentative substrates. This improved understanding will be key as we move forward with efforts to optimize ruminant livestock performance for the continued benefit of Mankind.

APPENDIX A: Influence of Two Rumen Cannulation Techniques on Postoperative Recovery in Sheep

This appendix section is published as: Schramm, H. H., Gleason, C. B., and White, R. R., 2020. Influence of two rumen cannulation techniques on postoperative recovery in sheep. Veterinary Surgery. 50:312-322.

ABSTRACT

Objective: To determine the influence of 2 methods of rumen cannulation on postoperative animal welfare.

Study Design: Experimental study.

Animals or Sample Population: Twelve commercial wethers (n = 6 per group).

Methods: Group 1 wethers underwent a 1-step procedure to place a cannula immediately after fistulation of the rumen to the skin. Group 2 wethers underwent a 2-step procedure where a portion of the rumen was externalized and held with a clamp for 9 days, after which the cannula was placed into the fistula created by removal of the clamped rumen tissue. Feed intake and vitals were monitored daily for 24 days postoperatively. Plasma fibrinogen and serum cortisol were measured daily for 14 days postoperatively to estimate inflammatory and stress responses, respectively. Change in body weight was also assessed.

Results: Cannulation method did not affect ($P > 0.05$) body weight, temperature, heart rate, respiratory rate, fibrinogen level, or cumulative cortisol level. Feed intake was lower (0.82 vs. 1.2 kg/d; $P < 0.0001$) and mean cortisol level was greater (124.2 vs. 121.5 ng/mL; $P = 0.038$) in Group 2 compared to Group 1.

Conclusion: Although both cannulation methods mostly elicited similar physiological responses, animals seemed to experience more discomfort and stress when undergoing the 2-step procedure.

Clinical Significance: Rumen cannulation performed in one step is recommended to improve postoperative welfare.

INTRODUCTION

Rumen fistulation in cattle, sheep, and goats has been performed extensively to advance our understanding of digestive physiology, nutrient degradability, and rumen microbial populations. Early work by Flourens (1833) described some of the first rumen fistulation procedures performed in sheep for studying rumen physiology. Since then, cannulation of the fistula has become standard to maintain the opening and reduce the impact of oxygen exposure on rumen function (Komarek, 1981). Two of the major surgical methods utilized by veterinary surgeons for rumen cannulation include the 1-step and 2-step techniques. The 1-step surgical procedure involves making a circular incision in the left paralumbar fossa and a subsequent incision in the rumen wall (Phillipson and Innes, 1939; Laflin and Gnad, 2008; Girardi et al., 2017). The rumen is sutured to the abdominal wall to form a fistula, into which a rubber or silicone cannula is then placed immediately after the procedure (Laflin and Gnad, 2008; Girardi et al., 2017). In the 2-step procedure, a portion of the rumen is externalized through a vertical incision in the left paralumbar fossa and held with a custom-made clamp to occlude blood flow and promote adhesion of the rumen to the incision edges of the abdominal wall (Schalk and Amadon, 1928). After approximately 7-10 days, the clamp and necrotic rumen tissue are removed to allow cannula placement into the now-formed fistula (Phillipson and Innes, 1939; Hecker, 1969; Martineau et al., 2015).

Despite numerous publications detailing these surgical methods and their variations, there is a lack of data available to compare postoperative responses between these methods. The relative implications of these procedures on animal welfare are therefore unknown. As animal welfare has come to the forefront as an important scientific and societal issue in recent decades, identification of the method with superior quality of life during postoperative recovery is

essential. The objective of this study was to investigate the influence of the 1- and 2-step rumen cannulation procedures on dry matter intake (**DMI**), body weight (**BW**), rectal temperature, heart rate, respiratory rate, cortisol levels, and fibrinogen levels in sheep to determine if one procedure had superior postoperative outcomes and improved animal welfare. We hypothesized that animals undergoing 1- and 2-step procedures would not differ in postoperative outcomes, including DMI, BW change, vital signs, and blood parameters. Through this study, our ultimate aim was to provide insight to surgeons and researchers regarding these 2 cannulation procedures.

MATERIALS AND METHODS

All procedures in this study were approved by the Virginia Tech Institutional Animal Care and Use Committee (Protocol #18-096).

Animals and Study Design

From a group of conspecifics, 12 commercial wethers (Suffolk, Dorset, or Suffolk x Dorset) were selected for use in the trial based on similarity in age, BW, breed, FAMACHA score, and normality of vital signs when screened by the herd health veterinarian. Animals were housed individually in indoor pens at the Virginia Tech Sheep Center, Blacksburg, VA. Wethers were approximately 16 months of age (range: 15-17 months) and 74.1 ± 7.8 kg BW (range: 62.3-84.1 kg) at study commencement. The diet consisted of alfalfa hay (90.6% dry matter, 16.7% crude protein, 48.7% neutral detergent fiber, 5.5% starch, 6.5% ash) fed at 2.5% BW in dry matter with loose minerals (Super Sheep Fortifier with Bovatec, Sweetlix, Mankato, MN) offered *ad libitum*. Clean, fresh water was available at all times. Wethers were stratified by initial BW, body condition score (**BCS**), and breed (defined as percent Suffolk) then randomly assigned to 1 of 2 surgery groups so that the means for BW, BCS, and breed percentage were similar

between groups. The 6 wethers within a surgery group were then randomly assigned to 1 of 2 surgery days so an equal number of 1- and 2-step procedures was performed each day. All surgeries were performed by the same veterinary surgeon.

Feeding, Monitoring, and Blood Analysis

Wethers were housed in pens 3-4 days prior to surgery for acclimation and to establish baseline measurements for DMI, vitals, and blood parameters. Alfalfa hay was provided twice daily at 0900 h and 1800 h and refusals collected to determine DMI. Heart rate, respiratory rate, and rectal temperature were recorded twice daily. Following surgery, surgical sites were inspected daily. A moistened gauze square was used to remove any debris or build-up around the surgical site and the area was subsequently wiped with dry gauze. Fly spray (Catron IV, Bayer, Leverkusen, Germany) was then applied. Veterinary intervention was sought if DMI fell below 1.5% of body weight for 3 days, if body temperature exceeded 40.8°C (105.5°F) for over 24 hours, or if respiratory rate exceeded 80 breaths/minute accompanied by fever above 40.8°C (105.5°F) for 4 hours. These physiological cutoffs were fairly high, by design, because the surgeries were performed during a particularly warm week in the summer.

Fibrinogen analysis. Blood was collected daily at 0800 h by intravenous jugular catheter and placed in a blood collection vial containing EDTA. Jugular catheters were placed at the same time in all animals, immediately prior to surgeries. Catheters were replaced every 4 days, or as needed, throughout the sampling period. Samples were refrigerated at 4°C until analysis (< 8 h). Fibrinogen levels were determined following the procedure used by the Virginia Tech Animal Laboratory Services (SOP# CP.HEMO.1-08, Version: 06.27.16). Briefly, blood was added to 2 capillary tubes and centrifuged for 60 seconds at 6,000 rpm (HemataSTAT-II, EKF Diagnostics,

Cardiff, United Kingdom). The plasma protein value of one tube for each animal was measured using a refractometer (Reichert TS Meter, Ametek Reichert Technologies, Depew, NY). A heated plasma protein value was measured similarly after heating the animal's second capillary tube at 56-58°C (132.8-136.4°F) for 3 minutes and re-centrifuging. Fibrinogen concentration was determined using the formula:

Fibrinogen (mg/dL) = Plasma Protein Value (g/dL) - Heated Plasma Protein Value (g/dL) x 1,000

Cortisol analysis. Blood was collected twice daily at 0800 and 1700 h by intravenous jugular catheter and placed in an anticoagulant-free blood collection vial. Samples were refrigerated at 4°C (39°F) overnight, then centrifuged at 2,000 rpm for 15 minutes to obtain serum. Serum was frozen at -18°C (0°F) until further analysis. Cortisol concentration (ng/mL) was determined by colorimetric assay following the procedure outlined in a commercially available cortisol ELISA kit (Cortisol [Sheep] ELISA Kit, Abnova, Taipei City, Taiwan). Sample absorbance was read at 450 nm on a microplate reader (Epoch 2 Microplate Spectrophotometer, BioTek, Winooski, VT).

Creation of the Acrylonitrile Butadiene Styrene Clamp

The acrylonitrile butadiene styrene (**ABS**) clamp (Figure 1A) used in the 2-step procedure was designed using the 3D computer-aided design tool Tinkercad (Autodesk, San Rafael, CA) and built by a 3D printer (Monoprice Maker Select Plus 3D Printer, Monoprice, Inc., Rancho Cucamonga, CA) using 1.75 mm ABS printer filament (Monoprice Premium 3D Printer Filament ABS 1.75 mm, Monoprice, Inc., Rancho Cucamonga, CA). The printing software used was Cura (Ultimaker, Geldermalsen, Netherlands), an open-source 3D printing

application available at https://ultimaker.com/en/products/ultimaker-cura-software. Clamp dimensions are shown in Figure 1B. This clamp is not commercially available.

Antibiotics, Analgesia, Sedation, and Preoperative Preparation

Feed was withheld for 12 hours prior to surgery to minimize rumen contractions during surgery and to facilitate exteriorization of the rumen. Access to free-choice water was maintained. Wethers were administered penicillin G procaine (PenOne Pro, MWI Animal Health, Boise, ID) and flunixin meglumine (Prevail, MWI Animal Health, Boise, ID) 2-4 hours prior to surgery at 27,000 IU/kg IM and 1.7 mg/kg IV, respectively. Penicillin G procaine administration was continued once daily for 3 days following the surgical procedure. Flunixin meglumine administration was continued once every 24 hours for 2 days. Wethers were administered a combination of 0.07 mg/kg ketamine (Zetamine, MWI Animal Health, Boise, Idaho), 0.03 mg/kg xylazine (Anased, MWI Animal Health, Boise, Idaho), and 0.02 mg/kg butorphanol (Torbugesic, Zoetis, Parsippany, New Jersey) IM for standing sedation.

The left paralumbar fossa was clipped from the dorsal midline to the flank fold and from just caudal to the tuber coxae cranially to the 10th intercostal space. Sheep were restrained on a trimming stand for the duration of the procedure with an assistant to hold them on the right side. The clipped area was cleaned and prepped with betadine scrub and isopropyl alcohol prior to administration of local anesthesia. A distal paravertebral nerve block was performed by infusing 5 mL of 1% lidocaine (Lidocaine, MWI Animal Health, Boise, Idaho) per site dorsal and ventral to the transverse processes of L1, L2, and L4 at a depth of 2.5-3.5 cm.

One-Step Surgical Procedure (Treatment 1)

A marking pen was used to superficially sketch the internal diameter of the cannula (7.6 cm) on the skin of the paralumbar fossa. A 1 inch, 18 gauge hypodermic needle was used to score the skin as a guide for the incision (Figure 2a). The clipped area was surgically prepared with betadine scrub and isopropyl alcohol. A 60 x 80 cm impervious drape was then placed over the surgical site. A circular incision approximately 7.6 cm in diameter was performed using a #10 scalpel and the skin was removed. The external abdominal oblique muscle was located and a circular area of muscle approximately 4 cm in diameter was removed at a central and slightly ventral location to the skin incision. The internal abdominal oblique muscle was then located and separated digitally for approximately 5-6 cm in length with the direction of the muscle fibers. A small vertical incision approximately 4-5 cm in length was then made using blunt dissection through the transversus abdominis muscle and peritoneum. The abdominal area near the incision was explored digitally to ensure peritonitis or adhesions were not present. The caudolateral aspect of the rumen, closest to the incision site, was located and exteriorized using Allis tissue forceps (Figure 2b). Two stay sutures (1 polyamide) were placed in the rumen and held by an assistant to keep the rumen exteriorized. Four temporary simple interrupted sutures (0 PDS) were placed at the 12, 3, 6, and 9 o'clock positions, tacking the skin to the rumen wall to prevent contamination of the abdomen and minimize tension on the rumen. A 3 cm vertical incision was made ventral to the 12 o'clock suture and the rumen was sutured to the skin using a simple continuous pattern with 1 polyamide suture. The rumen incision was extended as the suture pattern was extended ventrally to help prevent abdominal contamination. One quarter of the circumference was sutured at a time (12 to 3, 3 to 6, 6 to 9, and 9 to 12) and a new continuous pattern was started at each quarter (Figure 2c). The stay sutures were then removed. Once the

circular suture pattern was complete, the rumen cannula (#11C 2" Rubber Cannula, Bar Diamond, Parma, ID) was placed in the fistula (Figure 2d). The cannula was initially soaked in hot water prior to insertion to increase malleability for placement. A larger cannula (#8C 3" Rubber Cannula, Bar Diamond, Parma, ID) was fitted 9 days after surgery. A single dose of flunixin meglumine was administered to each animal prior to cannula size-up at 1.7 mg/kg IV. Sutures were removed 24 days postoperatively and a permanent adhesion confirmed. A permanent adhesion was defined as complete attachment of the rumen wall to the skin at the entire circumference of the incision (Figure 2e). Confirmation of an adhesion was an outcome variable and was not used to alter postoperative care in any way. The same veterinary surgeon evaluated the presence of adhesion in all animals.

Two-Step Surgical Procedure (Treatment 2)

A marking pen was used to sketch the location for the incision and placement of mattress sutures for this procedure (Figure 3a). A 1 inch 18 gauge needle was used to score the sketched locations for accuracy during surgery and to ensure correct location of the rumen cannula in the paralumbar fossa. The clipped area was surgically prepared with betadine scrub and isopropyl alcohol. A 60 x 80 cm impervious drape was then placed over the surgical site. A 6 cm vertical incision was made in the skin of the left paralumbar fossa 6-7 cm ventral to the transverse processes and 7-8 cm caudal to the last rib using a #10 scalpel. Three interrupted mattress sutures (3 polyamide suture) were placed on each side of the incision and were located 1 cm cranial and caudal to the incision. Hemostatic forceps were placed on the ends of each individual mattress suture (Figure 3b). The 3 hemostatic forceps on each side were held together using a Backhaus towel clamp fixed to the skin. Blunt dissection of the external abdominal oblique, internal abdominal oblique, and transverse abdominal muscles were performed along the muscle fibers

and the peritoneum was located. The peritoneum was then incised and digital exploration of the abdomen was completed to ensure no adhesions or peritonitis were present. The rumen wall was grasped with a pair of Allis tissue forceps near the center of the incision (caudolateral aspect of the rumen) and exteriorized. Two more Allis tissue forceps were placed on the rumen wall, one approximately 3 cm above the original forceps and one approximately 3 cm below the original forceps. An acrylonitrile butadiene styrene clamp was then placed over the incision with bolts pointing caudally. The forceps holding the rumen wall were passed through the clamp and the mattress sutures through the skin were threaded into the clamp slots. While the rumen was exteriorized between the internal edges of the clamp, an assistant gripped the clamp firmly as the bolts holding the clamp together were tightened. After ensuring all sutures remained within their respective slots and skin was not trapped within the clamp, it was tightened to prevent slippage of rumen tissue. A gap of 3-5 mm between the clamp edges was allowed (Figure 3c). The mattress sutures were then tied to secure the clamp against the abdominal wall. A 4 cm incision was made in the clamped portion of rumen and any digesta was flushed from this incision with sterile saline. Three to 6 unfolded gauze sponges were inserted into the cavity to prevent tissue slippage through the clamp jaws. The cavity was then closed using 1-0 catgut suture in an Utrecht pattern (Figure 3d). Clamp bolt ends were covered in adhesive tape to prevent skin irritation and the surgical site was disinfected with iodine solution. Nine days after placement, the externalized portion of rumen tissue was removed with a scalpel and the mattress sutures were removed to free the clamp. Successful adhesion between the rumen and abdominal wall was confirmed. The exterior portion of the fistula was cleaned with chlorhexidine solution and fitted with a rumen cannula (#11C 2” Rubber Cannula, Bar Diamond, Parma, ID). Manual stretching of the fistula was performed to stretch tissues to allow placement of a 7.6 cm cannula.

A single dose of flunixin meglumine was administered to each animal prior to cannula placement at 1.7 mg/kg IV. A larger cannula (#8C 3" Rubber Cannula, Bar Diamond, Parma, ID) was fitted 7 days later and a flunixin meglumine dose administered beforehand as above.

Statistical Analysis

Data were analyzed in R (R Statistical Software version 3.4.1, R Core Team, Vienna, Austria) using a mixed model approach with fixed effects of initial value and procedure. The response variables evaluated were BW change (kg), DMI (kg/d), rectal temperature (°C), heart rate (**BPM**), respiratory rate (**BrPM**), plasma fibrinogen (mg/dL), and serum cortisol (ng/mL). Day post-surgery was used as a repeated measure, and the interaction between procedure and day post-surgery was evaluated. Day of surgery was used as a random effect. All model derivations were performed using the R package nlme version 3.4.3 (Pinheiro et al., 2013). Significance was determined by analysis of variance (**ANOVA**). Significance level was set at $P < 0.05$ and a tendency considered when $0.05 \leq P < 0.10$.

Cortisol concentrations were analyzed in 2 ways. First, they were analyzed as a response over time, as described above. This analysis was intended to reflect whether one group differed from the other in terms of the mean cortisol readings. The area under the curve for cortisol responses was also analyzed because the timescales of the surgeries differed. This analysis was intended to better represent the cumulative exposure to elevated cortisol levels, considering the differing timelines for the expected stress responses.

RESULTS

The 1-step surgeries took from 65-96 minutes (median = 87.5 minutes) while the 2-step procedures ranged from 50-93 minutes in length (median = 67.5 minutes, and not including initial cannula placement 9 days later). Cannulation method did not affect mean BW change (-7.8 kg for the 1-step procedure vs. -9.6 kg for the 2-step procedure; $P = 0.23$), rectal temperature (39.6 vs. 39.5°C; $P = 0.11$), heart rate (85.5 vs. 84.8 BPM; $P = 0.96$), respiratory rate (49.5 vs. 46.3 BrPM; $P = 0.16$), or plasma fibrinogen (488.5 vs. 493.3 mg/dL; $P = 0.94$) (Table 1). Dry matter intake for the sheep that underwent the 2-step procedure was lower (0.82 vs. 1.2 kg/d; $P < 0.0001$) than for those receiving the 1-step procedure. Mean serum cortisol concentration was greater for the 2-step procedure (124.2 vs. 121.5 ng/mL; $P = 0.038$). However, cortisol levels were not different when analyzed cumulatively (38,494.44 vs. 36.163.82 ng/mL; $P = 0.82$). Examination of responses over time (Figures 4a-d and 4f,g) revealed a noticeable decrease in DMI (Figure 4a) and increases in rectal temperature (Figure 4b) and heart rate (Figure 4c) for the 2-step surgery group compared to the 1-step surgery group during days 9-11 of the postoperative period, corresponding to the time surrounding cannula size-up for the 1-step group and initial cannula placement for the 2-step group. Changes in respiratory rates over time (Figure 4d) were not different between groups. Ambient air temperatures (Figure 4e) appeared to possess no correlation with variations in respiratory rates (Figure 5). For both groups, plasma fibrinogen levels increased post-operatively then gradually declined (Figure 4f). A nonsignificant increase in serum cortisol concentration (Figure 4g) for the 2-step surgery group compared to the 1-step surgery group was observed during days 9-11 of the postoperative period. No dataset outliers were identified.

DISCUSSION

This study compared animal responses of weight loss, feed intake, vital signs, cortisol, and fibrinogen following a 1- or 2-step ruminal cannulation surgery. Overall, animals displayed similar responses on the basis of mean vital signs and fibrinogen levels but those receiving the 2-step procedure displayed decreased feed intake and increased average cortisol levels compared to those cannulated in 1 step. Increases in rectal temperature and heart rate were observed in animals in the 2-step group surrounding the time of clamp removal and initial cannula placement. These findings therefore did not unilaterally support our hypothesis that postoperative outcomes would not differ. Taken together, these recovery responses indicate the 2 surgical techniques offer generally comparable postoperative recovery experiences but that intake depression may occur when the 2-step method is used and that the activities of clamp removal and cannula fitting elicit relevant biological responses in the animal patients. The higher mean cortisol levels observed for the 2-step procedure suggest elevated stress and potential discomfort for these animals. A comparison of cumulative cortisol levels was also made in consideration of the differences in surgical timelines for the 2 treatment groups. However, the area under the cortisol curve for the 14 days post-surgery did not differ among treatment groups, implying that the cumulative stress response associated with post-operative recovery was similar among the treatments.

All animals ate immediately after surgery when presented with hay, regardless of surgical treatment. This is consistent with previous observations (Komarek, 1981; Elices Mínguez et al., 2010; Girardi et al., 2017). However, mean postoperative DMI was lower for the 2-step treatment group, declining steadily over time and failing to reach preoperative intake levels at the end of the experiment as was observed in the 1-step group (Figure 4a). A fairly dramatic (and

statistically significant) dip (-0.72 kg) in 2-step treatment DMI was also noted on day 9, corresponding to the day of clamp removal and initial cannula fitting. No such intake decrease was apparent for the 1-step treatment group that received a larger cannula on that day. This depression in intake could reflect increased stress or discomfort associated with the 2-step procedure. Alternatively, rumen motility may have been negatively affected by inflammation around the adhesion site due to stretching of the fistula to accommodate cannula insertion, which could have depressed intake. Animals receiving a 2-step cannulation may therefore benefit from interventions to help stimulate appetite and boost intake, such as providing concentrate feed, increasing feeding frequency, rumen transfaunation, and individual housing to reduce competition for feed. Cannula size-up on day 16 was not associated with a notable decrease in DMI (Figure 4a), suggesting it was a less disruptive event than clamp removal and initial cannula placement. Although 2-step animals lost numerically more weight than the 1-step treatment group (1.8 kg on average), the difference was not significant ($P = 0.23$; Table 1).

Vital signs represent the most basic body functions and their measurement is a very standard and simple way to assess the general health status of an individual. An abnormally high body temperature (pyrexia) can indicate the presence of an infection (Walter et al., 2016). Sterile inflammation and heat stress may also be associated with elevated body temperature. Animal rectal temperatures were variable over the course of the postoperative period, increasing post-surgery to d 10, plateauing, then declining after day 19 (Figure 4b). However, we observed no difference ($P = 0.11$; Table 1) in mean rectal temperatures of the 2 treatment groups nor did we observe any incision infections, suggesting that animals undergoing one technique may not be more prone to infections than those undergoing the other. No significant correlations were identified between rectal temperature and fibrinogen level or rectal temperature and ambient

temperature (Figure 5), demonstrating a lack of support that rectal temperature was associated with sterile inflammation or hot weather conditions. Increases in mean rectal temperature and heart rate for the 2-step group were observed during day 9-11, with day 9 being the day of clamp removal and initial cannula placement. These responses may have been due to stress or discomfort experienced during these activities. Mean heart rates and respiratory rates were not different between the treatments ($P = 0.96$ and $P = 0.16$, respectively; Table 1) during the postoperative period, however. To our knowledge, other comparisons of these surgical approaches have not monitored rectal temperatures, heart rates, or respiratory rates post-surgery so it is difficult to compare these results to previous work. Tachycardia and hyperventilation can both be exhibited in response to pain or stress (Muir, 2006; Gopinathannair and Olshansky, 2015; UPMC, 2019), which may be experienced during surgery recovery. Interestingly, mean heart rates increased steadily postoperatively while mean respiratory rates declined after surgery and remained low before increasing again from day 17 to day 23 (Figures 4c,d). While this period coincided with hot weather conditions (USDC/NOAA, 2018) (Figure 4e), no significant correlation was found between respiratory rates and ambient temperature (Figure 5). Variation in procedure-specific distress may therefore not have been adequately captured by vital parameters due to the effects of factors such as individual animal differences or day-to-day variation.

The blood glycoprotein fibrinogen performs essential functions in the processes of blood clotting and wound healing, as well as the inflammatory response (Mosesson, 2005). Mean blood fibrinogen levels were observed to increase post-surgery to d 6, then begin to gradually decline (Figure 4f). Increases in fibrinogen synthesis would be expected postoperatively as a response to the need for healing of the surgical incisions (Laurens et al., 2006). Mean fibrinogen levels did not differ between treatment groups ($P = 0.94$; Table 1), which suggests that neither surgical

treatment was associated with increased tissue damage and inflammatory response compared to the other. To our knowledge, no other studies have examined fibrinogen levels following cannulation surgery. Additional research will therefore be needed to provide a comparison for our observations.

The steroid hormone cortisol is a well-known and easily measured indicator of stress (Miller, 2008; Lee et al., 2015). Animals receiving the 2-step cannulation procedure displayed higher mean serum cortisol levels during the postoperative period than those receiving the 1-step treatment ($P = 0.038$; Table 1). Cumulative cortisol levels, however, were not different between treatment groups ($P = 0.82$). Examination of cortisol levels over time revealed numerically elevated cortisol levels for the Treatment 2 group during days 1 through 7 of the postoperative period (Figure 4G). During the second week of recovery, mean cortisol levels appear to be largely similar between the 2 treatment groups, with the exception of an increase observed in the 2-step animals during the time corresponding to clamp removal and initial cannula fitting. This difference was not significant, however. Considering these dynamics, it is understandable that a significant treatment effect could be seen for mean cortisol levels along with no effect on cumulative cortisol levels. Animals undergoing 2-step cannulation might therefore be more stressed at specific points during surgery recovery than animals cannulated in 1 step, but the 2 surgical procedures may be expected to elicit a similar cumulative stress response overall. A treatment group was handled whenever the other was receiving a procedure, so the bias of handling-induced stress was minimized. Cortisol levels were also not likely impacted by seasonal variation due to the trial's timeframe and the fact that indoor housing was utilized. As with vital signs, the current literature appears to lack reports of cortisol levels in animals having

undergone either method of cannulation surgery. Future investigations will be necessary to provide equivalent data to relate to our findings.

Rumen clamps required for the 2-step technique are not commercially available currently, prompting researchers to design their own, typically out of metal (Hecker, 1969) or wood (Martineau et al., 2015). We used a 3D printed ABS clamp with success. None of the clamps became dislodged and there were no instances of a clamped rumen portion coming free and falling back into the body, as had been previously reported when wooden clamps were used in cattle (Martineau et al., 2015). Stronger ruminal contractions in cattle may perhaps exert more strain on the clamps compared to sheep rumens. It may also indicate that ABS is more effective at preventing slippage of tissues than wood. Martineau and colleagues (2015) reported additional complications after cannula insertion (which was performed 7 days post-surgery) due to incomplete rumen-abdominal wall adhesion. No failures to reach complete adhesion were noted in our 2-step surgery group, indicating that it may be beneficial to delay cannula placement by an additional 2-3 days to allow for more complete adhesion of the tissues. Peritonitis and incisional infections have been reported as complications of the 1-step procedure (Hartnack et al., 2015) but were not observed in any of our wethers receiving either surgical treatment.

Conclusions

In this research, we offered a novel comparison of the 2 major cannulation methods currently used in ruminant nutrition research on the basis of postoperative recovery experience for the animal patient. Animals receiving the 2-step cannulation method displayed decreased feed intake compared to those undergoing the 1-step method. Mean cortisol concentrations were also higher for the 2-step cannulation group. While differences in vital signs were not significant,

animals receiving the 2-step method experienced biologically relevant increases in body temperature and heart rate on the day of clamp removal and initial cannula placement. These findings lead us to conclude that the 2 surgeries elicit relevant differences in the animals they are performed on, with the 2-step method resulting in greater overall stress. Animal welfare considerations may therefore support usage of the 1-step procedure over the 2-step. Animals that do undergo the 2-step method may require attention to their feed intake and benefit from extra efforts to limit discomfort during the postoperative period. These measures should help to improve animal welfare in such research endeavors. Further research is necessary to elucidate any long-term effects of cannulation method on animal health and well-being.

LITERATURE CITED

Elices Mínguez, R., L. Revuelta Rueda, J. Martínez-Darve, and S. Miguélez Fernández. 2010. Surgery technique for ovine ruminal cannulation/Procedimiento quirúrgico para la implantación de una fistula permanente en ovejas. Rev. Complut. Cienc. Vet. 4:41–52.

Flourens, M. 1833. Experiences sur le mechanisme de la rumination. Mem Acad Roy Sci Inst Fr. 12:483.

Girardi, A. M., S. S. Sousa, A. F. Sabes, G. M. Bueno, T. J. C. Módolo, Y. da S. Bonacin, B. de A. Pimenta, J. A. Marques, and L. C. Marques. 2017. One-stage rumen fistulation with permanent silicone cannula in sheep. Nucl. Anim. 9:91–96. doi:10.3738/21751463.2140.

Gopinathannair, R., and B. Olshansky. 2015. Management of tachycardia. F1000Prime Rep. 7. doi:10.12703/P7-60. Available from: http://f1000.com/prime/reports/m/7/60

Hartnack, A. K., A. J. Niehaus, M. Rousseau, R. L. Pentecost, M. D. Miesner, and D. E. Anderson. 2015. Indications for and factors relating to outcome after rumenotomy or rumenostomy in cattle: 95 cases (1999–2011). J. Am. Vet. Med. Assoc. 247:659–664. doi:10.2460/javma.247.6.659.

Hecker, J. F. 1969. A simple rapid method for inserting rumen cannulae in sheep. Aust. Vet. J. 45:293–294. doi:10.1111/j.1751-0813.1969.tb01954.x.

Komarek, R. J. 1981. Rumen and abomasal cannulation of sheep with specially designed cannulas and a cannula insertion instrument. J. Anim. Sci. 53:790–795. doi:10.2527/jas1981.533790x.

Laflin, S. L., and D. P. Gnad. 2008. Rumen cannulation: Procedure and use of a cannulated bovine. Vet. Clin. North Am. Food Anim. Pract. 24:335–340. doi:10.1016/j.cvfa.2008.02.007.

Laurens, N., P. Koolwijk, and M. P. M. De Maat. 2006. Fibrin structure and wound healing. J. Thromb. Haemost. 4:932–939. doi:10.1111/j.1538-7836.2006.01861.x.

Lee, D. Y., E. Kim, and M. H. Choi. 2015. Technical and clinical aspects of cortisol as a biochemical marker of chronic stress. BMB Rep. 48:209–216. doi:10.5483/BMBRep.2015.48.4.275.

Martineau, R., J. G. Proulx, C. Côrtes, A. F. Brito, and T. F. Duffield. 2015. Two-stage rumen cannulation technique in dairy cows. Vet. Surg. 44:551–556. doi:10.1111/vsu.12318.

Miller, W. L. 2008. Steroidogenic enzymes. In: C. E. Flück and W.L. Miller, editors. Disorders of the human adrenal cortex. Vol. 13. Karger Publishers, Basel, Switzerland. p. 1–18. Available from: https://www.karger.com/Article/FullText/134751

Mosesson, M. W. 2005. Fibrinogen and fibrin structure and functions. J. Thromb. Haemost. 3:1894–1904. doi:10.1111/j.1538-7836.2005.01365.x.

Muir, W. 2006. Trauma: physiology, pathophysiology, and clinical implications. J. Vet. Emerg. Crit. Care. 16:253–263. doi:10.1111/j.1476-4431.2006.00185.x.

Phillipson, A. T., and J. R. M. Innes. 1939. Permanent stomach fistulae in ruminants. Q. J. Exp. Physiol. Cogn. Med. Sci. 29:333–341. doi:10.1113/expphysiol.1939.sp000812.

Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar, and R Core Team. 2013. nlme: Linear and nonlinear mixed effects models. R Foundation for Statistical Computing, Vienna, Austria.

Schalk, A. F., and R. S. Amadon. 1928. Physiology of the ruminant stomach (bovine): Study of the dynamic factors. Agricultural Experiment Station, North Dakota Agricultural College.

UPMC. 2019. Tachycardia: Causes, symptoms, and treatment. Univ. Pittsburgh Med. Cent. Heart Vasc. Inst. Available from: https://www.upmc.com/services/heart-vascular/conditions-treatments/tachycardia

USDC/NOAA. 2018. Record of climatological observations. Natl. Cent. Environ. Inf. Available from: https://www.ncdc.noaa.gov/data-access/land-based-station-data/data-publications

Walter, E. J., S. Hanna-Jumma, M. Carraretto, and L. Forni. 2016. The pathophysiological basis and consequences of fever. Crit. Care. 20:200. doi:10.1186/s13054-016-1375-5.

TABLE

Table 1. Summary of Results: LS Means ± SEM

Variable	1-Step procedure	2-Step procedure	*P*-value
Body weight change, kg	-7.8 ± 1.1	-9.6 ± 1.2	0.23
Dry matter intake, kg/d	1.2 ± 0.10	0.82 ± 0.11	<0.0001
Rectal temperature, °C	39.6 ± 0.07	39.5 ± 0.07	0.11
Heart rate, BPM	85.5 ± 2.4	84.8 ± 2.4	0.96
Respiratory rate, BrPM	49.5 ± 3.3	46.3 ± 3.3	0.16
Plasma fibrinogen, mg/dL	488.5 ± 39.5	493.3 ± 39.4	0.94
Serum cortisol, ng/mL	121.5 ± 7.7	124.2 ± 7.7	0.038
Cumulative serum cortisol, ng/mL	38,494.44	36,163.82	0.82

FIGURES

Figure 1a. Acrylonitrile butadiene styrene rumen clamp with bolts and nuts.

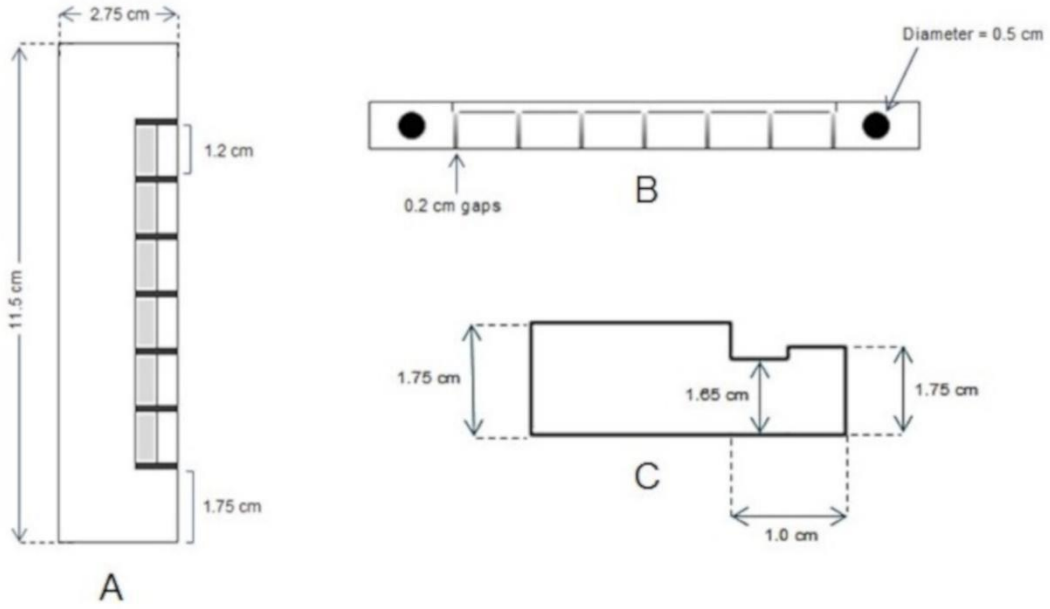

Figure 1b. Clamp dimensions. A: front view; B: lateral view; C: cross section.

Figure 2a. The skin of the paralumbar fossa is scored with a 1 inch, 18 gauge hypodermic needle as a guide for the incision.

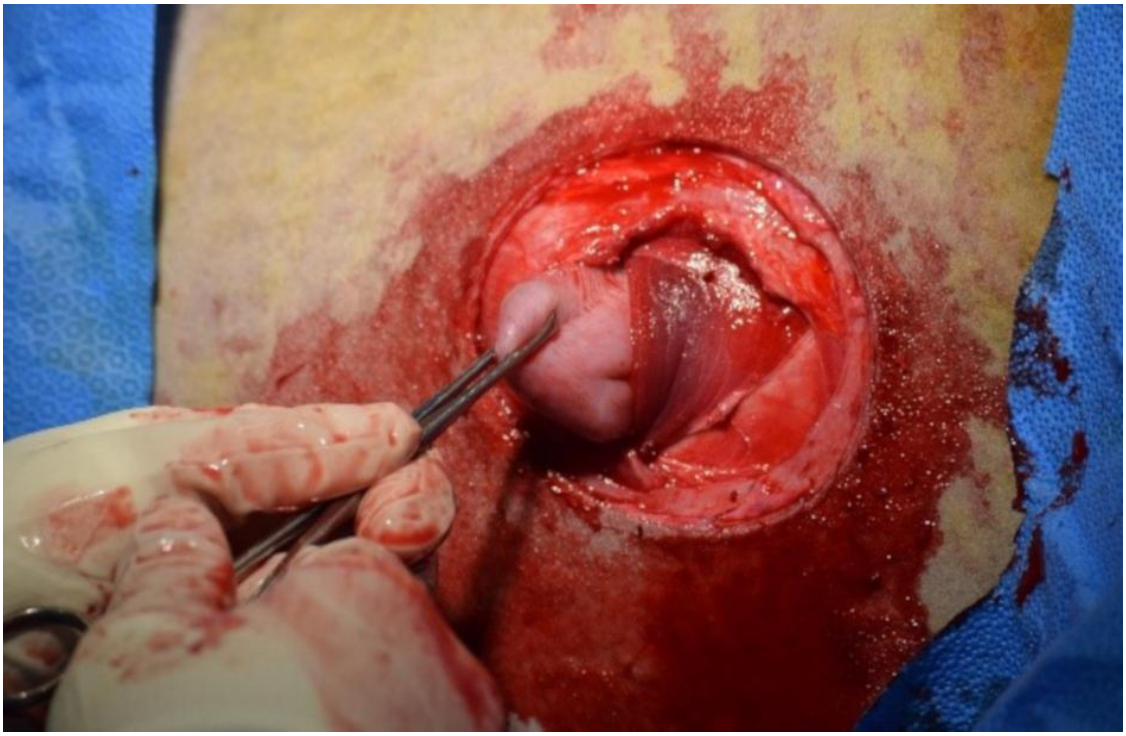

Figure 2b. The caudolateral aspect of the rumen, closest to the incision site, is located and exteriorized using Allis tissue forceps.

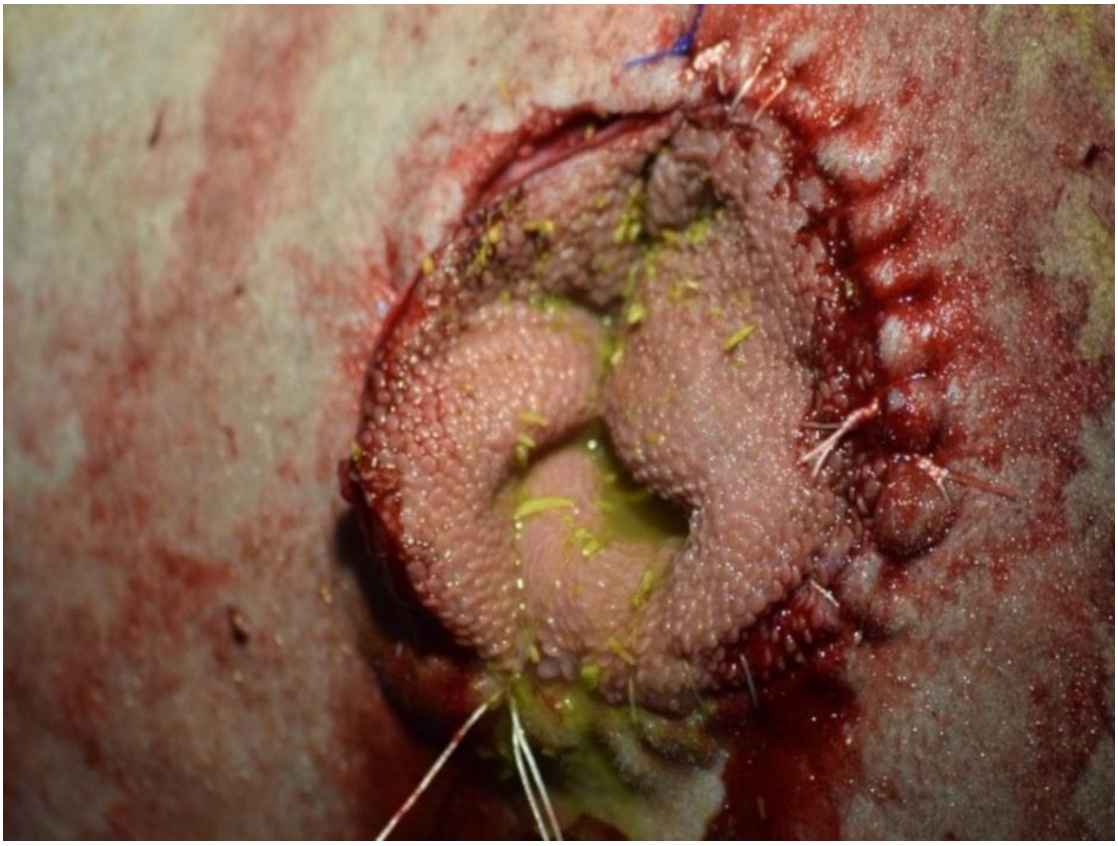

Figure 2c. One quarter of the incision's circumference is sutured at a time and a new continuous suture pattern is started at each quarter.

Figure 2d. The rubber cannula is placed in the fistula following completion of the circular suture pattern.

Figure 2e. Example of a permanent adhesion between rumen tissue and skin.

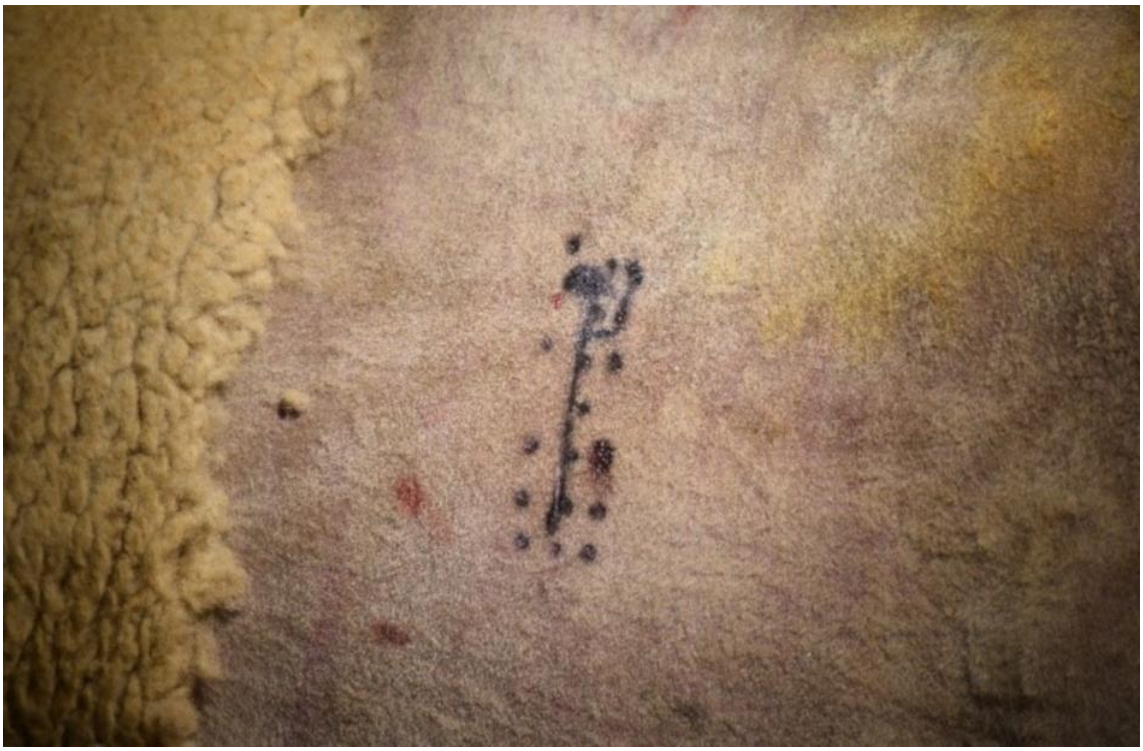

Figure 3a. The location for the incision and placement of mattress sutures for this procedure are sketched using a marking pen.

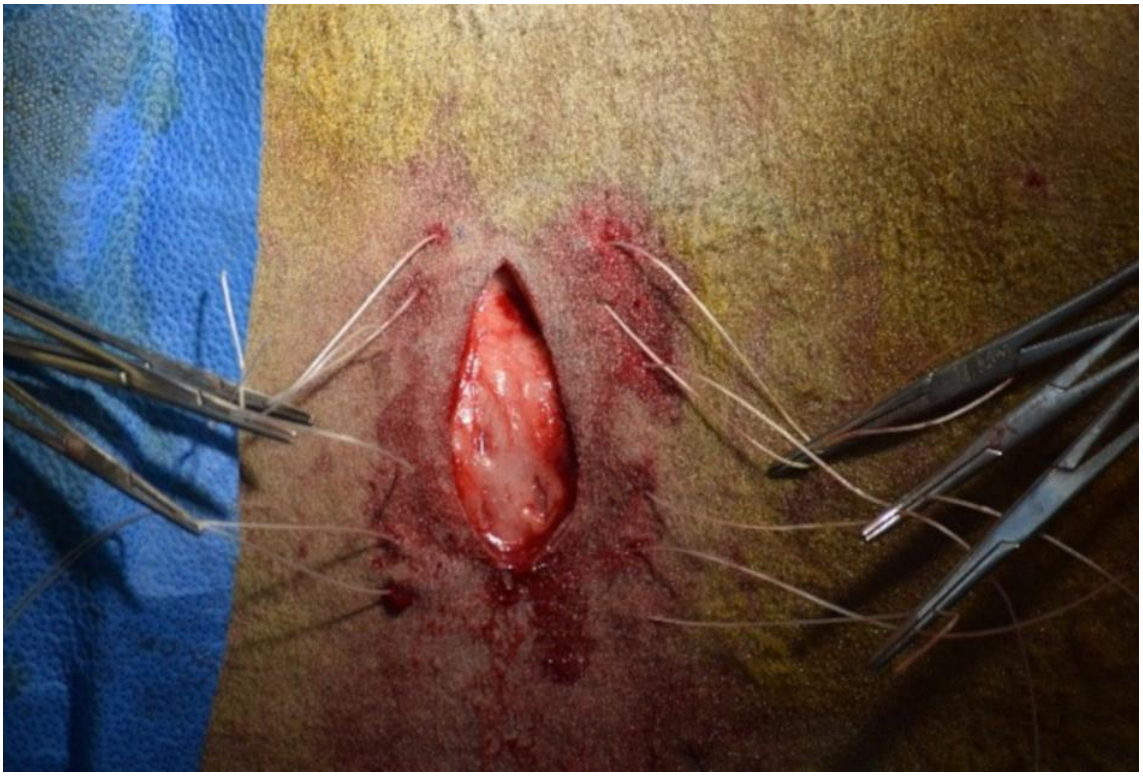

Figure 3b. Hemostatic forceps are placed on the ends of each mattress suture.

Figure 3c. After ensuring all sutures remain within their respective slots and no skin is trapped, the clamp is tightened on the exteriorized portion of rumen tissue to allow a gap of 3-5 mm between the clamp's edges.

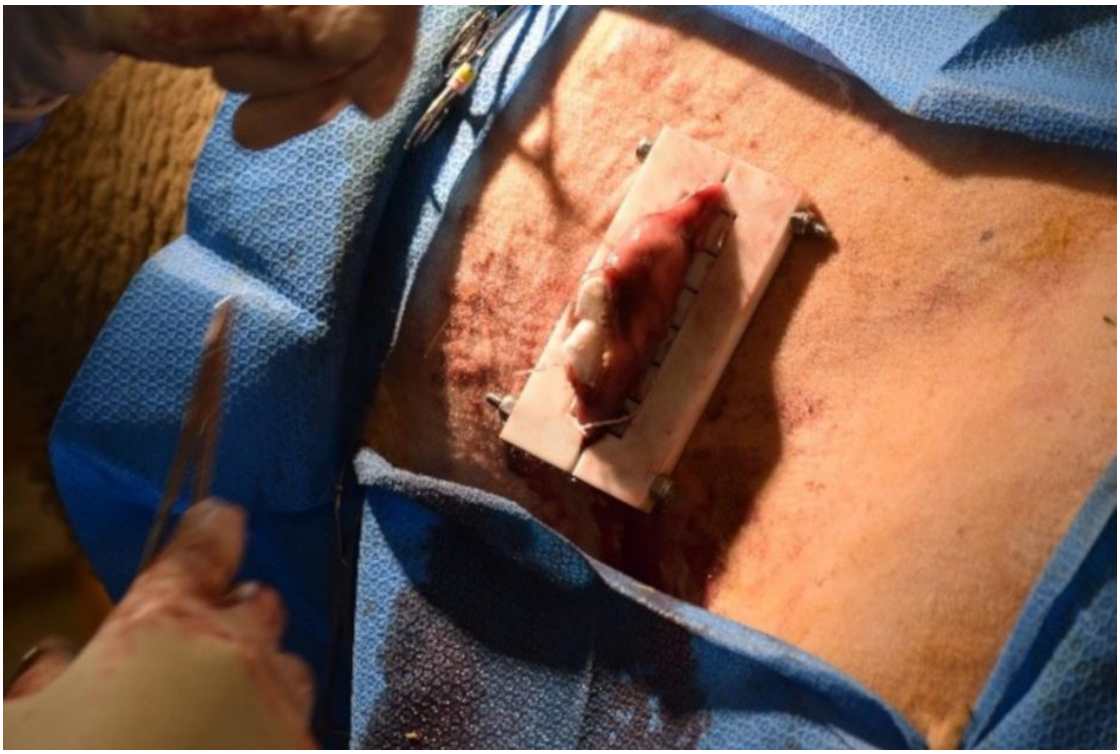

Figure 3d. Three to 6 unfolded gauze sponges were inserted into the cavity created by incising the clamped, exteriorized rumen tissue. The incision was then closed using 1-0 catgut suture in an Utrecht pattern.

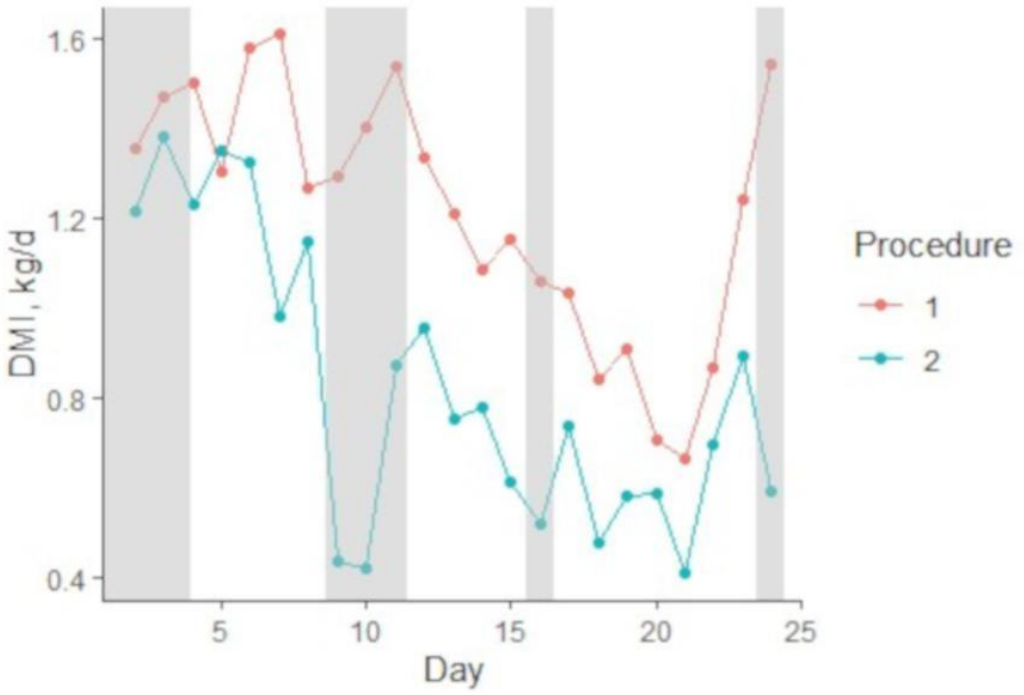

Figure 4a. Change in dry matter intakes. Procedure 1 corresponds to the 1-step procedure and Procedure 2 to the 2-step. Gray bars represent days 0-3, days 9-11, day 16, and day 24 of the postoperative period. Surgeries were performed on day 0. Cannula size-up (1-step) and insertion (2-step) occurred day 9. Cannula size-up (2-step) occurred day 16 and suture removal (1-step) occurred day 24.

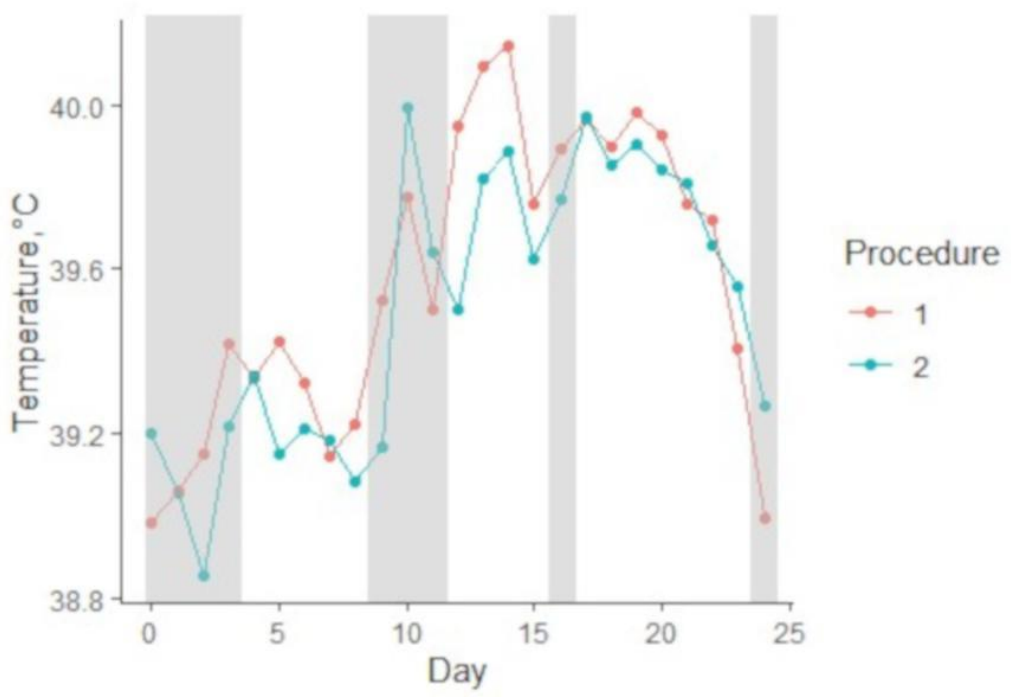

Figure 4b. Change in rectal temperatures. Procedure 1 corresponds to the 1-step procedure and Procedure 2 to the 2-step. Gray bars represent days 0-3, days 9-11, day 16, and day 24 of the postoperative period. Surgeries were performed on day 0. Cannula size-up (1-step) and insertion (2-step) occurred day 9. Cannula size-up (2-step) occurred day 16 and suture removal (1-step) occurred day 24.

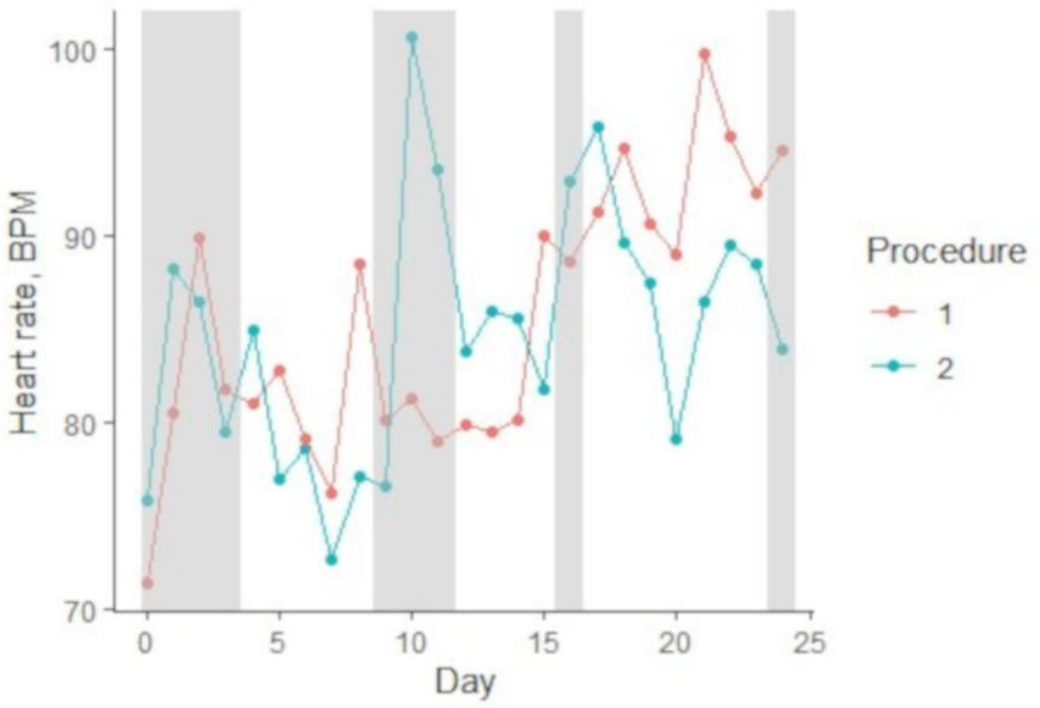

Figure 4c. Change in heart rates. Procedure 1 corresponds to the 1-step procedure and Procedure 2 to the 2-step. Gray bars represent days 0-3, days 9-11, day 16, and day 24 of the postoperative period. Surgeries were performed on day 0. Cannula size-up (1-step) and insertion (2-step) occurred day 9. Cannula size-up (2-step) occurred day 16 and suture removal (1-step) occurred day 24.

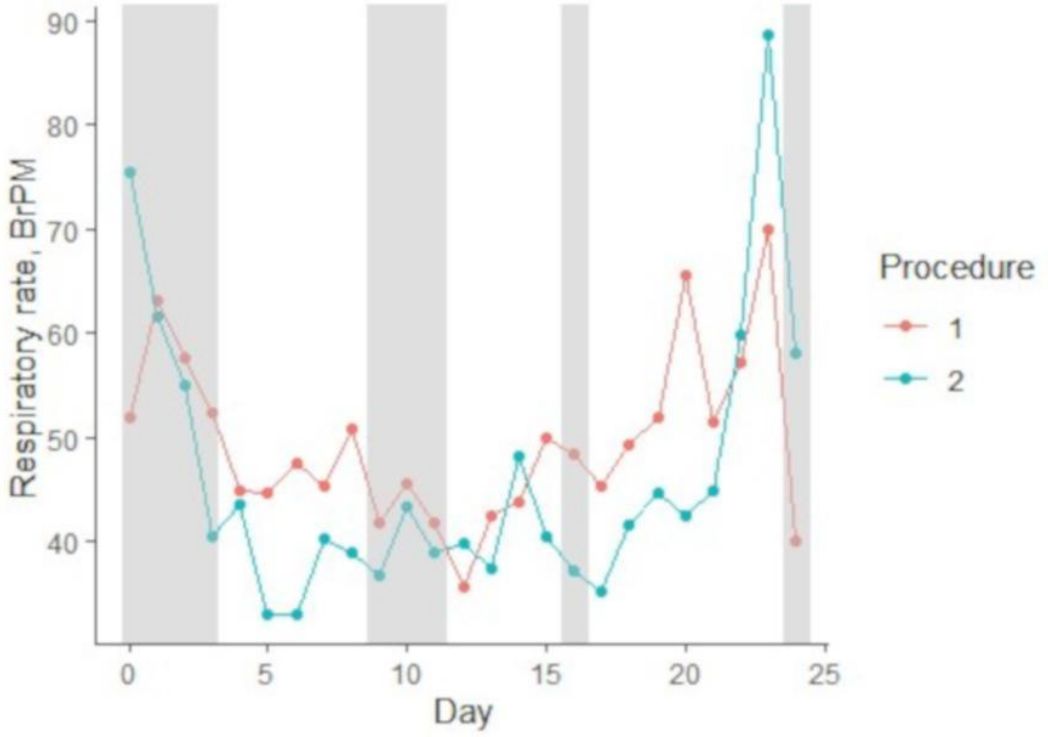

Figure 4d. Change in respiratory rates. Procedure 1 corresponds to the 1-step procedure and Procedure 2 to the 2-step. Gray bars represent days 0-3, days 9-11, day 16, and day 24 of the postoperative period. Surgeries were performed on day 0. Cannula size-up (1-step) and insertion (2-step) occurred day 9. Cannula size-up (2-step) occurred day 16 and suture removal (1-step) occurred day 24.

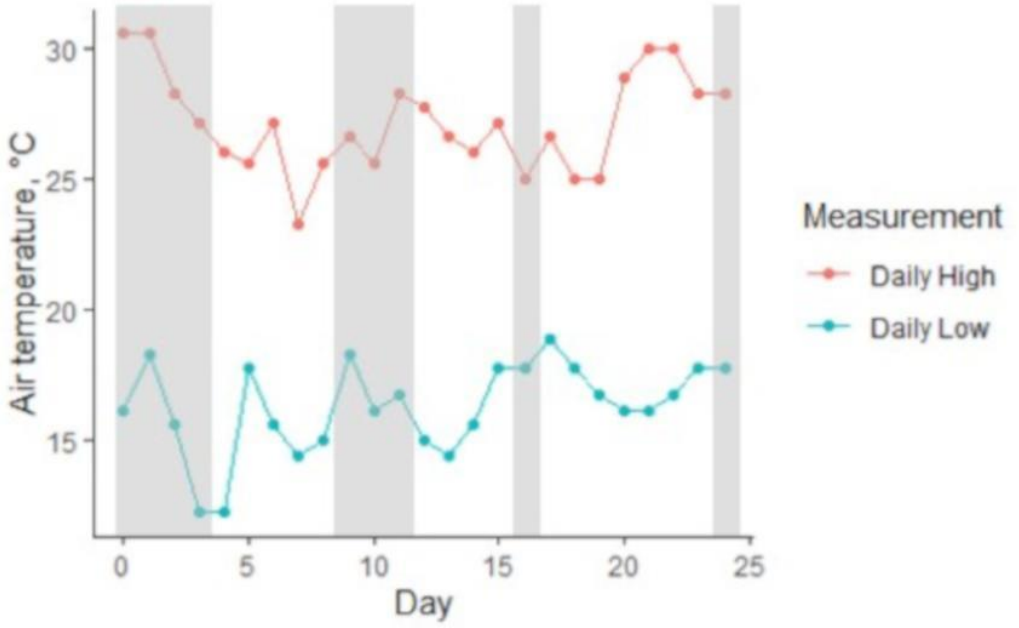

Figure 4e. Change in average air temperature (data from NOAA-NCEI, 2019). Gray bars represent days 0-3, days 9-11, day 16, and day 24 of the postoperative period. Surgeries were performed on day 0. Cannula size-up (1-step) and insertion (2-step) occurred day 9. Cannula size-up (2-step) occurred day 16 and suture removal (1-step) occurred day 24.

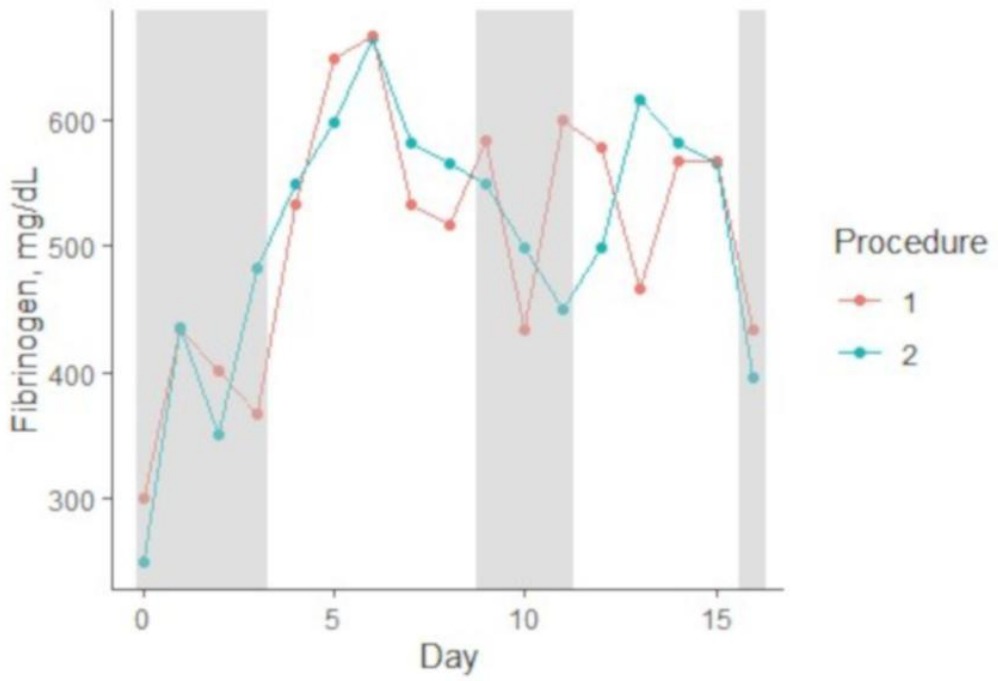

Figure 4f. Change in plasma fibrinogen concentrations. Procedure 1 corresponds to the 1-step procedure and Procedure 2 to the 2-step. Gray bars represent days 0-3 and days 9-11 of the postoperative period. Surgeries were performed on day 0. Cannula size-up (1-step) and insertion (2-step) occurred day 9.

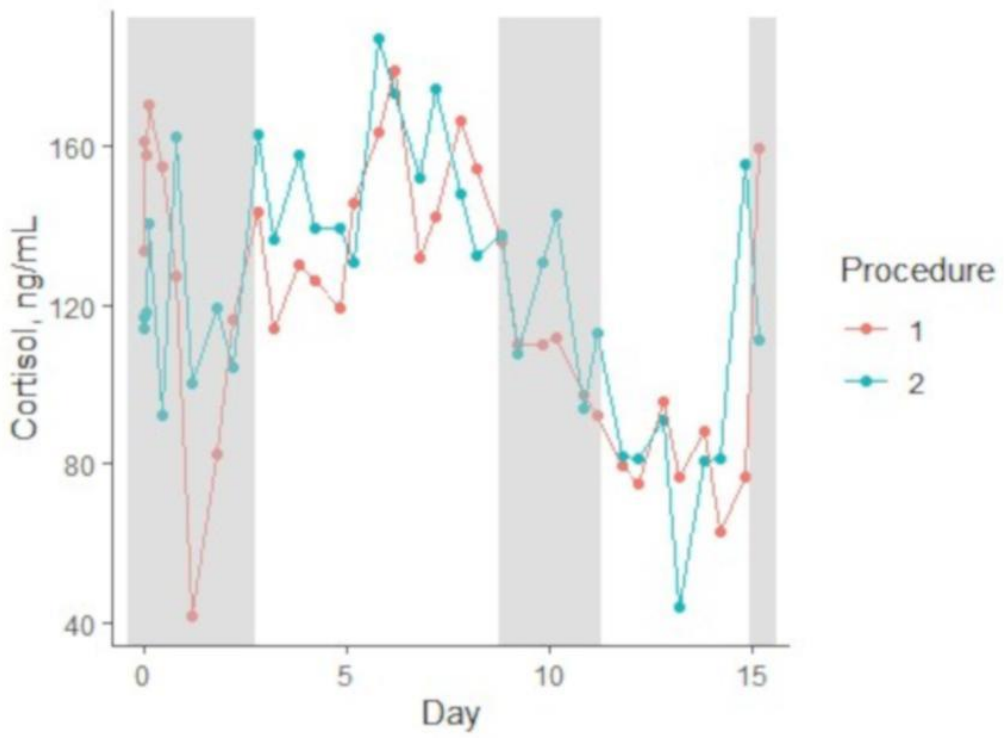

Figure 4g. Change in serum cortisol concentrations. Procedure 1 corresponds to the 1-step procedure and Procedure 2 to the 2-step procedure. Gray bars represent days 0-3, days 9-11, and day 16 of the postoperative period. Surgeries were performed on day 0. Cannula size-up (1-step) and insertion (2-step) occurred day 9. Cannula size-up (2-step) occurred day 16.

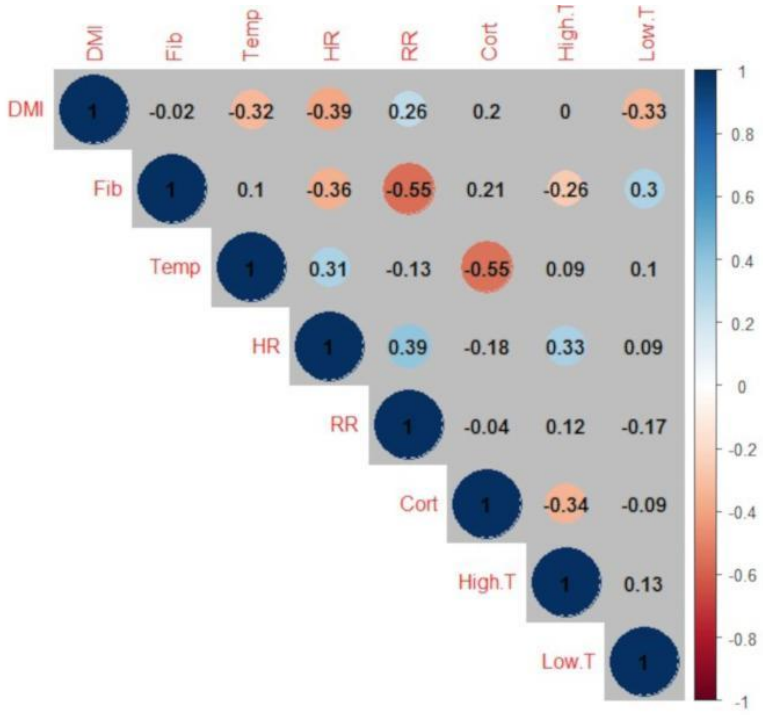

Figure 5. Correlations among variables measured. DMI = dry matter intake, Fib = fibrinogen, Temp = rectal temperature, HR = heart rate, RR = respiratory rate, Cort = cortisol, High.T = high ambient temperature, Low.T = low ambient temperature. Colored circles indicate significant correlations. Numbers reflect the Pearson correlation coefficient.

APPENDIX B: Evaluation of Rumen Modifiers to Limit Pellet Intake in Beef Brood Cows

This appendix section will be published as: Gleason, C. B., T. B. Wilson, V. R. G. Mercadante, A. Bedford, K. L. Herkelman, and R. R. White. Evaluation of rumen modifiers to limit pellet intake in beef brood cows. Journal of Animal Science.

ABSTRACT

Winter supplementation of gestating beef cows is often necessary to ensure energy and protein requirements remain satisfied. However, it is difficult to prevent over- or under-consumption by individual animals fed in a group. The objective of this study was to evaluate the intake limiting effects of 3 levels of tea saponin (**TS**) on pelleted feed consumption when compared with a TS-free control treatment and to identify the level of TS that could most effectively limit dry matter intake to 1.5% of body weight (**DMIBW**). Commercial beef cows in late gestation (n = 24) were allocated to 1 of 4 treatments delivered via a pelleted feed supplement: 0% (**A**), 0.16% (**B**), 0.32% (**C**), or 0.64% (**D**) TS on a dry matter basis. Cows were assigned so that initial mean body weights and body condition scores were similar among treatment groups. Supplement was delivered once daily via Calan gates at a beginning rate of 2.0% of BW for 42 d. Refusals were collected daily to calculate intake. Treatment differences were observed for pellet DMI, cow BW, and cow BCS ($P < 0.0001$). Cow hay intake, calf birth weight, and calf weaning weight were unaffected by treatment ($P > 0.05$). Dry matter intake of pellets as a percent of BW was significantly different for all treatments ($P < 0.0001$) with intake declining as TS content increased. Considerable variability in DMIBW of all treatments was observed from Day 0 to 15 but intakes plateaued between approximately 1.75 and 2.5% DMIBW for the remainder of the trial with Treatment D intake remaining noticeably lower than the other treatments. Treatment D was found to be successful at limiting pellet intake to an average DMIBW of 1.5%. This study concluded that short-term pellet intake can be limited by inclusion of TS, highlighting it as a potential intake limiter product for beef cattle producers.

Keywords: beef cattle, feed intake, rumen modifiers, winter supplementation

INTRODUCTION

The impacts of bovine maternal nutrient status during gestation on offspring health and performance are well documented (Corah et al., 1975; Greenwood and Cafe, 2007; Larson et al., 2009; Long et al., 2010). Nutrient status of pregnant females influences growth characteristics (Houghton et al., 1990; Martin et al., 2007) as well as carcass quality of progeny (Underwood et al., 2010). Nutrient restriction during gestation has been found to negatively impact immunity status of offspring (McGee et al., 2006). Inadequate prepartum nutrition is also known to contribute to longer postpartum intervals and reduced pregnancy rates of the dams (Joubert, 1954; Selk et al., 1988). Supplementation is therefore occasionally required to address nutritional deficiencies in gestating females, particularly when they are to be maintained on low quality forage during the winter months. A common challenge for beef producers, however, is delivering a target amount of energy or protein supplement to individual gestating cows. Because group housing is standard, it is difficult to prevent over- or underconsumption of a provided supplement and nearly impossible to ensure that each animal consumes a specific target amount of feed. Fortunately, the use of an intake limiter can help ensure that each animal consumes only a target amount of supplement relative to its body weight. Common limiters include salt, gypsum, calcium chloride, and phosphoric acid. Although salt is a reasonably safe and inexpensive limiter, utilization of the other compounds can be accompanied by the challenges of sulfur toxicity (gypsum), corrosivity (calcium chloride), and expensive handling requirements (phosphoric acid) (Kunkle et al., 2000).

Saponins are bitter-tasting compounds found in plants that reduce plant palatability in high enough concentrations (Cheeke, 1996). Structurally, they are glycosides of either steroidal or triterpenoid aglycones with variable numbers of sugar side chains (Foerster, 2006). To date,

the effects of saponins on feed intake and dietary preferences of ruminants have received little scientific attention. Applications of saponins in ruminant nutrition have focused instead on their antimicrobial properties and abilities to optimize rumen fermentation and enhance nutrient utilization (Benchaar et al., 2008; Patra and Saxena, 2009; McMurphy et al., 2014b). The central objective of this study was therefore to evaluate the intake limiting effects of 3 levels of tea saponin (**TS**), a triterpenoid saponin isolated from *Camellia sinensis* (the tea plant), on pelleted supplement consumption compared with a TS-free control supplement and to identify the level of TS that could most effectively limit intake to a target of 1.5% in dry matter intake as a percent of body weight (**DMIBW**). Secondary objectives were to evaluate the effects of differing levels of limiter treatment on cow BW, cow BCS, forage intake, calf birth weight, and calf weaning weight.

MATERIALS AND METHODS

Animals and Study Design

All procedures in this study were approved by the Virginia Tech Institutional Animal Care and Use Committee (Protocol #17-164). Twenty-four commercial beef cows (BW = 537 ± 33 kg) of predominantly Angus genetics were maintained in a 1.33 ha pasture at Virginia Tech Kentland Farm, Blacksburg, VA. All cows were 210 days pregnant to timed artificial insemination at trial commencement. Cows were stratified by initial BW and BCS then randomly assigned to 1 of 4 supplemental treatments so that each group contained 6 cows. Treatments (Table 1) were custom grain pellets manufactured by Cooperative Research Farms (North Chesterfield, VA) containing varying levels of TS: 0% (**A**), 0.16% (**B**), 0.32% (**C**), or 0.64% (**D**). Treatments were delivered to cows daily via the Calan Broadbent Feeding System (American

Calan Inc., Northwood, NH) at a rate of 2.5% of BW in DM for 42 d. Calan feeders were mounted onto 2 covered flatbed trailers with 6 feeders on each side. Trailers were parked in a 13 x 25 m lot adjacent to the pasture and cows had 24 h access to the Calan feeders. First-cutting grass hay (Table 1), minerals, and water were available *ad libitum*.

Data Collection

Refusals were collected and weighed each day at 0800 h to calculate pellet DMI, after which the daily allotment of supplement was weighed and delivered. Cows were weighed on d 0, 14, 28, and 42 using a digital scale (Tru-Test ID5000, Carbine Aginvest, Auckland, New Zealand) located under a squeeze chute. Cows were body condition scored immediately after weighing using a scale of 1 to 9, with 1 being emaciated and 9 being obese. Calves were born approximately 30 d after trial completion and calf birth weights were taken within 24 h of birth. Calves were weaned and weighed at 6 mo of age. Titanium dioxide was utilized as an external marker for estimation of forage DMI and was added to the pellets during milling at a concentration of 200 g/T. A fecal sample (approximately 100 g) was collected rectally from each cow every 6 h starting on d 38 for a total of 9 collection times evenly distributed over the day. Samples were dried for 36 h at 55°C in a forced-air oven (Thermo Scientific Heratherm Advanced Protocol Oven Model 51028115, Fisher Scientific, Waltham, MA) and ground to pass through a 1 mm screen of a Wiley mill (Model 4, Thomas Scientific, Swedesboro, NJ). Samples were pooled by animal, ashed in a muffle furnace (Sybron Thermolyne FA1730, Fisher Scientific, Waltham, MA) for 12 h at 500°C, and digested in concentrated sulfuric acid. Titanium concentrations were determined on an inductively coupled plasma atomic emission spectrometer (SPECTRO ARCOS SOP, SPECTRO Analytical Instruments, Inc., Kleve, Germany). Estimated hay DMI was calculated following the method of de Souza et al. (2015).

Nutrient Analysis

One sample of each treatment pellet was collected at the end of the study. Hay samples were collected twice weekly and pooled to create a representative sample. Pellet and hay samples were dried at 55°C in a forced-air oven (Thermo Scientific Heratherm Advanced Protocol Ovens Model 51028115, Fisher Scientific, Waltham, MA) for 24 h and ground to pass through a 1 mm screen of a Wiley mill (Model 4, Thomas Scientific, Swedesboro, NJ). Dry matter content was determined by drying for 12 h at 100°C. Ash percent was assessed through combustion in a muffle furnace (Sybron Thermolyne FA1730, Fisher Scientific, Waltham, MA) for 12 h at 500°C. Neutral detergent fiber and ADF concentrations were determined using the Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY). Alpha-amylase from *Bacillus licheniformis* (Thermostable Amylase HTL, BIO-CAT, Troy, VA) and sodium sulfite (Sodium Sulfite, Anhydrous, Fisher Scientific, Waltham, MA) were utilized in the NDF analysis (Van Soest et al., 1991). Acid detergent lignin content was obtained by agitating ADF residues in 72% sulfuric acid on a rocking platform (Flask Dancer, Boekel Scientific, Feasterville-Trevose, PA) for 3 h (AOAC, 2000). Samples were combusted using a Vario El Cube CN analyzer (Elementar Americas Inc., Mount Laurel, NJ) to measure N content. Crude protein concentration was then calculated as %N × 6.25. Starch content was assessed following the acetate buffer method described by Hall (2015) with α-amylase and amyloglucosidase from *Aspergillus niger* (E-AMGDF, Megazyme International, Wicklow, Ireland). *In vitro* dry matter digestibility (**DMD**) was performed on each feed sample by Cumberland Valley Analytical Services (Waynesboro, PA).

Statistical Analysis

All data were analyzed in R version 3.4.1 (R Core Team, 2017) using a linear mixed-effects model and the nlme package (Pinheiro et al., 2013). Response variables included pellet DMI, cow BW, pellet DMIBW, cow BCS, cow forage intake, calf birth weight, and calf weaning weight. Treatment was used as a fixed effect while cow and Calan feeder trailer (1 of 2) were included as random effects. A 1st order autoregressive residual error variance structure was used for each of the response variables. Analysis of variance was performed on each model and least square means calculated. Significance was considered when $P < 0.05$ and a tendency when $0.05 \leq P < 0.10$.

RESULTS AND DISCUSSION

Pellet and Forage Intake

Least square means for each treatment are given in Table 2. As anticipated, pellet DMI was affected by TS concentration, with cows on the highest limiter percentage (0.64%) consuming 3.3 kg less pellet on average than those receiving the control with no limiter (Table 2). Pellet DMI of the lowest TS percentage (0.16%) tended ($P = 0.066$) to be lower than control pellet DMI. All TS concentrations resulted in significantly different pellet DMIBW compared to the control, with pellet DMIBW decreasing with increasing TS concentration (Table 2). With a mean pellet DMIBW of 1.51%, Treatment D (0.64% TS) was closest at limiting pellet intake to the target of 1.5% of BW. Interestingly, estimated grass hay intake was not different ($P = 0.85$) between the treatment groups despite the differences observed in pellet intake. This was consistent with the observations of McMurphy et al. (2014a), who noted no effect of the saponin from *Yucca schidigera* (known as sarsaponin or Micro-Aid commercially) on hay DMI in spring-

calving beef cows. This indicates that cows restricted from high pellet consumption due to the limiter's effects did not compensate by increasing their hay intake.

Few reports of saponin effect on feed intake in cattle are present in the literature, and no published studies utilizing saponin sourced from *Camellia sinensis* in beef brood cows appear to exist. In dairy cows, however, TMR intake has been shown to decrease in response to inclusion of sarsaponin in a number of studies (Wu et al., 1994; Lovett et al., 2006; Benchaar et al., 2008). Reported saponin concentrations were similar to or lower than those used in the present study: 0.31% (Lovett et al., 2006), 0.28% (Benchaar et al., 2008), and 0.007% (Wu et al., 1994). McMurphy et al. (2014b) reported that administration of sarsaponin to beef steers fed a protein supplement and low quality hay did not affect DMI ($P = 0.40$). However, the sarsaponin dosages administered were 1 and 2 g/d, which may have been insufficient to stimulate a significant intake response in these animals. Our pellet intake results therefore appear to be consistent with previous work where comparable saponin levels were utilized.

Limiter Efficacy Over Time

A considerable degree of variation in pellet DMIBW was noted for all treatments from Day 0 to 15 of the trial (Fig 1). This large degree of variation may have been due to the cows responding to the presence of the limiter and becoming accustomed to its effects, which can include gastrointestinal irritation (Wen et al., 2015). An instance of initial overeating may have led to internal discomfort, prompting the cow to decrease pellet intake the following day before increasing consumption again when the effects subsided, and potentially explains the intake variability observed during the beginning of the trial. After Day 15, mean pellet intakes on all treatments plateaued between approximately 1.75 and 2.5% DMIBW for the rest of the trial.

Final pellet DMIBW of all TS treatments was therefore greater than the desired target of 1.5% DMIBW. This loss of limiter efficacy was likely the result of the cows developing a tolerance to the TS treatment. Cattle are also known to increase their intake of salt limiters over time, prompting the need to increase the limiter dosage to maintain intakes at desirable levels (Kunkle et al., 2000). Successful long-term applications of saponin limiters will likely require dosage increases as well. Although also increasing from low levels initially, mean pellet DMIBW of Treatment D remained visibly lower compared to the other 3 treatments and averaged the target value of 1.5% DMIBW across time, indicating that a concentration of 0.64% TS is likely sufficient to limit intake for short feeding periods.

Body Weight, Body Condition Score, Calf Birth Weight, and Calf Weaning Weight

Both mean BW and BCS increased with increasing TS concentration (Table 2). Mean BW on all TS treatments were significantly different from the control and from each other. These significant differences were also observed for BCS. Calf birth weights were not different between treatments ($P = 0.15$) so did not likely contribute to the differences in cow BW. Although reports on saponin effects on weight change and body condition in adult cows are scarce, our BW and BCS results are consistent with those of McMurphy et al. (2014a), who also noted BW and BCS increases in gestating cows in response to sarsaponin supplementation. McMurphy et al. (2014a) also reported no treatment effect on calf birth weights or weaning weights, in agreement with our findings.

Further comparisons with the literature are challenging due to the deficiency of beef brood cow data, however, a number of studies report similar findings with saponin applications in other ruminant models. Mader and Brumm (1987), for instance, observed both an increase in

ADG and feed efficiency in growing beef steers supplemented with sarsaponin. Greater ADG was also reported in sheep by several studies where saponins from *Enterolobium cyclocarpum* (Leng et al., 1992; Navas-Camacho et al., 1993) or *Sapindus saponaria* (Hess et al., 2004) were utilized. As mentioned, saponins have been studied in cattle for their desirable manipulations of rumen function. The increased level of condition (and by extension BW) seen in our cows receiving higher TS concentrations may have been partially attributed to enhanced fermentation and digestibility, and therefore greater energy uptake by these animals. This could not be confirmed, however, because the cows were not cannulated and total-tract digestibility was not assessed, being outside the scope of the project. The higher degree of body fat observed in high-limiter cows may also have contributed to the decrease in feed consumption through the intake regulatory effects of released leptin or unsaturated fatty acids (NRC, 1987). Although mean BCS scores were higher for the cows receiving higher limiter dosages, they are still considered suboptimal (<5; Table 2). Decreased palatability of the TS-containing diets may therefore have been a larger contributor to the lower pellet DMI observed.

Broader Implications of Saponin Utilization

The major action of saponins in the rumen is depopulation of protozoa (Patra and Saxena, 2009). This defaunation results in a decrease in bacterial proteolysis, which improves N conservation and increases efficiency of microbial protein synthesis (McMurphy et al., 2014b). There is evidence that saponins impose selective pressure on bacterial populations and archaeal activities as well (Wang et al., 2000; Hess et al., 2003; Guo et al., 2008). Methanogens have demonstrated decreased methane production, but not total numbers, in response to saponin administration (Hess et al., 2003). This may be at least partially attributed to the defaunating effect of the treatment, which would remove protozoal support of methane production. Tea

saponin has also been shown to reduce methyl coenzyme-M reductase subunit A gene expression and methane production in cultured rumen methanogens (Guo et al., 2008). Wang and colleagues (2000) noted that sarsaponin negatively impacted cellulolytic bacterial populations but did not affect amylolytic bacteria, indicating a useful application of saponin in high concentrate diets. In agreement with this finding, a number of studies report lower ruminal acetate:propionate ratios on saponin treatments of varying sources (Lu and Jorgensen, 1987; Hristov et al., 1999; Abreu et al., 2004; Hess et al., 2004; Santoso et al., 2007). Dietary inclusion of saponins has also been observed to improve rumen degradability of DM and NDF (attributed to a slower particulate passage rate) as well as increase microbial protein flow to the small intestine (McMurphy et al., 2014b). Taken together, these effects on the rumen environment may collectively promote efficiency of energy and nitrogen usage through the favoring of metabolically efficient pathways, activities, and participants. Our results appear to reflect this logic because, despite consuming less pelleted supplement and a similar amount of hay, cows on higher TS treatments were able to maintain a more desirable BCS with no difference in calf birth weights. The inclusion of saponin in the diets of beef cattle may therefore provide a number of metabolic, production, and environmental benefits beyond simply limiting intake.

Conclusions

This study offered a novel demonstration of the intake-limiting effects of tea saponin in gestating beef brood cows as well as its impacts on forage consumption, body weight, body condition, calf birth weight, and calf weaning weight. A saponin concentration of 0.64% was found to be sufficient to limit supplement intake to a target of 1.5% of body weight. The observation of increased tolerance to saponin administration prompts further investigation of strategies to maintain a target supplement intake long-term, likely involving an increase in

limiter dosage over time. The added benefits of saponins in optimizing rumen function and nutrient utilization underscore the potential value of tea saponin as a commercial product for beef cattle producers.

LITERATURE CITED

Abreu, A., J. E. Carulla, C. E. Lascano, T. E. Diaz, M. Kreuzer, and H.-D. Hess. 2004. Effects of Sapindus saponaria fruits on ruminal fermentation and duodenal nitrogen flow of sheep fed a tropical grass diet with and without legume. J. Anim. Sci. 82:1392–1400. doi:10.2527/2004.8251392x.

Benchaar, C., T. A. McAllister, and P. Y. Chouinard. 2008. Digestion, ruminal fermentation, ciliate protozoal populations, and milk production from dairy cows fed cinnamaldehyde, quebracho condensed tannin, or Yucca schidigera saponin extracts. J. Dairy Sci. 91:4765–4777. doi:10.3168/jds.2008-1338.

Cheeke, P. R. 1996. Biological effects of feed and forage saponins and their impacts on animal production. In: Saponins used in food and agriculture. Springer. p. 377–385.

Corah, L. R., T. G. Dunn, and C. C. Kaltenbach. 1975. Influence of prepartum nutrition on the reproductive performance of beef females and the performance of their progeny. J. Anim. Sci. 41:819–824. doi:10.2527/jas1975.413819x.

Foerster, H. 2006. Soybean saponin I biosynthesis. BioCyc. Available from: https://biocyc.org/META/NEW-IMAGE?type=PATHWAY&object=PWY-5203&detail-level=3

Greenwood, P. L., and L. M. Cafe. 2007. Prenatal and pre-weaning growth and nutrition of cattle: long-term consequences for beef production. Animal. 1:1283–1296. doi:10.1017/S175173110700050X.

Guo, Y. Q., J.-X. Liu, Y. Lu, W. Y. Zhu, S. E. Denman, and C. S. McSweeney. 2008. Effect of tea saponin on methanogenesis, microbial community structure and expression of mcrA gene, in cultures of rumen micro-organisms. Lett. Appl. Microbiol. 47:421–426. doi:10.1111/j.1472-765X.2008.02459.x.

Hall, M. B. 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method: Collaborative study. J. AOAC Int. 98:397–409. doi:10.5740/jaoacint.15-012.

Hess, H.-D., R. A. Beuret, M. Lötscher, I. K. Hindrichsen, A. Machmüller, J. E. Carulla, C. E. Lascano, and M. Kreuzer. 2004. Ruminal fermentation, methanogenesis and nitrogen utilization of sheep receiving tropical grass hay-concentrate diets offered with Sapindus saponaria fruits and Cratylia argentea foliage. Anim. Sci. 79:177–189. doi:10.1017/S1357729800054643.

Hess, H.-D., L. M. Monsalve, C. E. Lascano, J. E. Carulla, T. E. Diaz, and M. Kreuzer. 2003. Supplementation of a tropical grass diet with forage legumes and Sapindus saponaria fruits: Effects on in vitro ruminal nitrogen turnover and methanogenesis. Aust. J. Agric. Res. 54:703–713. doi:10.1071/AR02241.

Houghton, P. L., R. P. Lemenager, L. A. Horstman, K. S. Hendrix, and G. E. Moss. 1990. Effects of body composition, pre- and postpartum energy level and early weaning on reproductive

performance of beef cows and preweaning calf gain. J. Anim. Sci. 68:1438–1446. doi:10.2527/1990.6851438x.

Hristov, A. N., T. A. McAllister, F. H. Van Herk, K.-J. Cheng, C. J. Newbold, and P. R. Cheeke. 1999. Effect of Yucca schidigera on ruminal fermentation and nutrient digestion in heifers. J. Anim. Sci. 77:2554–2563. doi:10.2527/1999.7792554x.

Joubert, D. M. 1954. The influence of high and low nutritional planes on the oestrous cycle and conception rate of heifers. J. Agric. Sci. 45:164–172. doi:10.1017/S0021859600046712.

Kunkle, W. E., J. T. Johns, M. H. Poore, and D. B. Herd. 2000. Designing supplementation programs for beef cattle fed forage-based diets. J. Anim. Sci. 77. doi:10.2527/jas2000.00218812007700ES0012x.

Larson, D. M., J. L. Martin, D. C. Adams, and R. N. Funston. 2009. Winter grazing system and supplementation during late gestation influence performance of beef cows and steer progeny. J. Anim. Sci. 87:1147–1155. doi:10.2527/jas.2008-1323.

Leng, R. A., S. H. Bird, and A. Klieve. 1992. Legume trees and other fodder trees as protein sources for livestock. In: P.-L. Pugliese and A. Speedy, editors. Proceedings of the FAO Expert Consultation held at the Malaysian Agricultural Research and Development Institute. FAO, Kuala Lumpur, Malaysia.

Long, N. M., M. J. Prado-Cooper, C. R. Krehbiel, U. DeSilva, and R. P. Wettemann. 2010. Effects of nutrient restriction of bovine dams during early gestation on postnatal growth, carcass and organ characteristics, and gene expression in adipose tissue and muscle. J. Anim. Sci. 88:3251–3261. doi:10.2527/jas.2009-2512.

Lovett, D. K., L. Stack, S. Lovell, J. Callan, B. Flynn, M. Hawkins, and F. P. O'Mara. 2006. Effect of feeding Yucca schidigera extract on performance of lactating dairy cows and ruminal fermentation parameters in steers. Livest. Sci. 102:23–32. doi:10.1016/j.livsci.2005.11.005.

Lu, C. D., and N. A. Jorgensen. 1987. Alfalfa saponins affect site and extent of nutrient digestion in ruminants. J. Nutr. 117:919–927. doi:10.1093/jn/117.5.919.

Mader, T. L., and M. C. Brumm. 1987. Effect of feeding sarsaponin in cattle and swine diets. J. Anim. Sci. 65:9–15. doi:10.2527/jas1987.6519.

Martin, J. L., K. A. Vonnahme, D. C. Adams, G. P. Lardy, and R. N. Funston. 2007. Effects of dam nutrition on growth and reproductive performance of heifer calves. J. Anim. Sci. 85:841–847. doi:10.2527/jas.2006-337.

McGee, M., M. J. Drennan, and P. J. Caffrey. 2006. Effect of age and nutrient restriction pre partum on beef suckler cow serum immunoglobulin concentrations, colostrum yield, composition and immunoglobulin concentration and immune status of their progeny. Ir. J. Agric. Food Res. 157–171.

McMurphy, C. P., A. J. Sexten, G. L. Mourer, M. J. Rincker, and D. L. Lalman. 2014a. Effects of including saponins (Micro-Aid®) in a protein supplement on performance of growing steers and spring-calving cows. Anim. Feed Sci. Technol. 190:19–29. doi:10.1016/j.anifeedsci.2014.01.002.

McMurphy, C. P., A. J. Sexten, G. L. Mourer, E. D. Sharman, S. J. Trojan, M. J. Rincker, W. K. Coblentz, and D. L. Lalman. 2014b. Effects of including saponins (Micro-Aid®) on intake, rumen fermentation and digestibility in steers fed low-quality prairie hay. Anim. Feed Sci. Technol. 190:47–58. doi:10.1016/j.anifeedsci.2014.01.007.

Navas-Camacho, A., M. A. Laredo, A. Cuesta, H. Anzola, and J. C. Leon. 1993. Effect of supplementation with a tree legume forage on rumen function. Livest. Res. Rural Dev. 5:60–74.

NRC. 1987. Predicting feed intake of food-producing animals. National Academies Press, Washington, D.C.

Patra, A. K., and J. Saxena. 2009. The effect and mode of action of saponins on the microbial populations and fermentation in the rumen and ruminant production. Nutr. Res. Rev. 22:204–219. doi:10.1017/S0954422409990163.

Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar, and R Core Team. 2013. nlme: Linear and nonlinear mixed effects models. R Foundation for Statistical Computing, Vienna, Austria.

R Core Team. 2017. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Santoso, B., A. Kilmaskossu, and P. Sambodo. 2007. Effects of saponin from Biophytum petersianum Klotzsch on ruminal fermentation, microbial protein synthesis and nitrogen utilization in goats. Anim. Feed Sci. Technol. 137:58–68. doi:10.1016/j.anifeedsci.2006.10.005.

Selk, G. E., R. P. Wettemann, K. S. Lusby, J. W. Oltjen, S. L. Mobley, R. J. Rasby, and J. C. Garmendia. 1988. Relationships among weight change, body condition and reproductive performance of range beef cows. J. Anim. Sci. 66:3153. doi:10.2527/jas1988.66123153x.

de Souza, J., F. Batistel, K. C. Welter, M. M. Silva, D. F. Costa, and F. A. Portela Santos. 2015. Evaluation of external markers to estimate fecal excretion, intake, and digestibility in dairy cows. Trop. Anim. Health Prod. 47:265–268. doi:10.1007/s11250-014-0674-6.

Underwood, K. R., J. F. Tong, P. L. Price, A. J. Roberts, E. E. Grings, B. W. Hess, W. J. Means, and M. Du. 2010. Nutrition during mid to late gestation affects growth, adipose tissue deposition, and tenderness in cross-bred beef steers. Meat Sci. 86:588–593. doi:10.1016/j.meatsci.2010.04.008.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597. doi:10.3168/jds.S0022-0302(91)78551-2.

Wang, Y., T. A. McAllister, L. J. Yanke, and P. R. Cheeke. 2000. Effect of steroidal saponin from Yucca schidigera extract on ruminal microbes. J. Appl. Microbiol. 88:887–896. doi:10.1046/j.1365-2672.2000.01054.x.

Wen, L., N. Xia, P. Tang, Y. Hong, Z. Wang, YaJie Liu, YanJu Liu, J. Liu, and X. Li. 2015. The gastrointestinal irritation of Polygala saponins and Its potential mechanism in vitro and in vivo. BioMed Res. Int. 2015:1–8. doi:10.1155/2015/918048.

Wu, Z., M. Sadik, F. T. Sleiman, J. M. Simas, M. Pessarakli, and J. T. Huber. 1994. Influence of yucca extract on ruminal metabolism in cows. J. Anim. Sci. 72:1038–1042. doi:10.2527/1994.7241038x.

TABLES

Table 1. Nutrient composition of supplemental pellets and grass hay

Item, %[1,2]	Pellet A	Pellet B	Pellet C	Pellet D	Grass hay
DM	92.1	92.3	91.2	91.2	90.7
CP	16.3	16.2	16.7	17.0	13.5
NDF	44.8	42.9	45.5	46.5	67.7
ADF	24.0	23.1	24.6	24.4	37.7
ADL	1.53	1.71	1.46	1.71	4.76
Starch	23.0	25.3	26.2	22.5	3.01
Ash	7.57	8.69	6.65	6.77	8.75
TS	0.00	0.16	0.32	0.64	0.00
In vitro DMD	75.1	74.2	75.0	74.1	52.8

[1]Percentages are expressed on a DM basis except for DM, which is expressed on an AF basis.
[2]TS = tea saponin; DMD = dry matter digestibility.

Table 2. Least square means for treatment groups[1,2,3]

Item	A	B	C	D	SE	*P*-values for contrasts					
						A − B	A − C	A – D	B – C	B – D	C − D
Pellet DMI, kg	13.2[a]	12.6[a]	11.3[b]	9.90[c]	0.20	0.066	<0.0001	<0.0001	0.011	<0.0001	0.0001
Pellet DMIBW, %	2.21[a]	2.01[b]	1.79[c]	1.51[d]	0.69	0.0006	<0.0001	<0.0001	0.011	<0.0001	<0.0001
Hay DMI, kg	16.8 [a]	18.6 [a]	19.9 [a]	14.5[a]	4.5	0.99	0.96	0.98	>0.99	0.91	0.82
Mean cow BW, kg	555[a]	572[b]	581[c]	601[d]	19	0.0005	<0.0001	<0.0001	0.23	<0.0001	0.0002
Initial cow BW, kg	542[a]	532[a]	538[a]	541[a]	13	0.94	>0.99	>0.99	0.99	0.97	>0.99
Final cow BW, kg	564[a]	600[b]	601[b]	619[c]	14	<0.0001	<0.0001	<0.0001	0.99	<0.0001	<0.0001
Mean cow BCS	4.08[a]	4.26[b]	4.39[c]	4.63[d]	0.14	0.0008	<0.0001	<0.0001	0.92	0.001	0.008
Initial cow BCS	4.17[a]	4.41[a]	4.27[a]	4.29[a]	0.23	0.83	0.99	0.97	0.95	0.97	>0.99
Final cow BCS	4.00[a]	4.38[b]	4.13[c]	4.76[d]	0.14	<0.0001	0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Calf birth weight, kg	36.0[a]	36.7[a]	32.6[a]	33.3[a]	1.4	0.98	0.34	0.55	0.20	0.36	0.98
Calf weaning weight, kg	281 [a]	248 [a]	274 [a]	271 [a]	13	0.34	0.99	0.96	0.53	0.62	>0.99

[1]Means bearing different superscript letters differ significantly ($P < 0.05$).

[2]Pellet A contained 0.00% tea saponin (TS), B contained 0.16% TS, C contained 0.32% TS, and D contained 0.64% TS.

[3]SE = standard error; DMIBW = dry matter intake as a percent of body weight; BCS = body condition score.

FIGURE

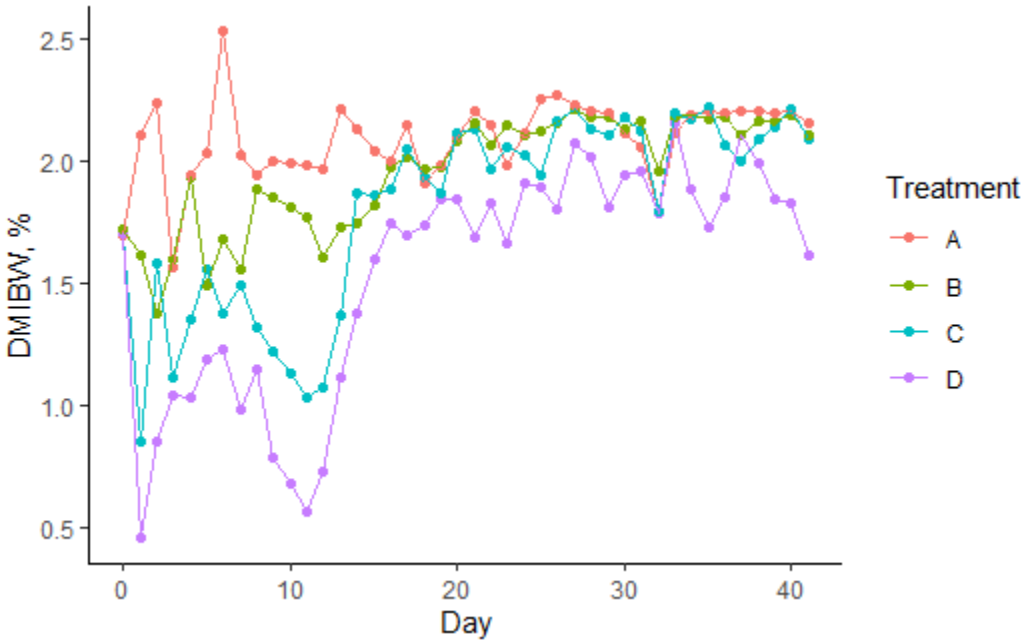

Figure 1. Change in pellet dry matter intake as a percent of body weight (DMIBW) over time.