Alternative Analytical and Experimental Procedures to Explore Rumen Fermentation as Driven by Nutrient Supplies

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ABSTRACT

Ruminant livestock play a vital role in fulfilling the nutrient requirements of humans by providing protein, energy, and essential microminerals. With the increasing demand for meat and dairy products, the ruminant industry must continue to improve the productivity and efficiency of ruminant animals with limited resources while minimizing the environmental impact. Rumen fermentation is the focal point of the productivity and efficiency of the animal and numerous chemical, physical and biochemical interactions make the rumen a complex ecosystem. Therefore, improving the understanding of fermentation dynamics in a holistic manner and characterizing how fermentation varies in response to different nutrient supplies can greatly expand our knowledge on rumen fermentation to develop better engineered rumen manipulation strategies. The central aim of these investigations was to employ alternative analytical strategies for holistic exploration of complex relationships among rumen, animal, and dietary variables and to estimate rumen volatile fatty acid (VFA) dynamics under different nutrient supplies. The objective of the first study was to explore the strengths and limitations of mixed-model meta-analysis, recursive feature elimination (RFE), and additive Bayesian networking (ABN) in identifying relationships among diet, rumen, and milk performance variables. Both mixed-models and ABN agreed upon most of the variables and relationships identified while RFE failed to capture interactions. Given the capacity of mixed models for quantitative inquiry and the potential of ABN to illustrate complex associations in a more intuitive way, future investigations combining both approaches hold potential to explore intercorrelated data in a holistic manner. Followed by the successful use of ABN in the first study, the goal of a follow up study was to investigate the potential of two different network approaches to explore rumen level interactions using data generated in continuous culture experiments. Two network analysis approaches, EBIC-LASSO network (ELN) and Bayesian learning network (BLN) were leveraged to explore the relationships among rumen fermentation parameters in continuous culture experiments. Unidirectional ELN illustrated prominent variables while BLN, which produces a directed acyclic graph, identified directional relationships implying causality. Overall, both networking approaches demonstrate strengths in capturing connectedness and directionality of rumen fermentation variables. In a complementary line of work, the next experiment focused on developing an alternative method for iso-tope based assessments to produce less expensive, and more efficient screening of fermentation conditions driven by diet. Cannulated wethers were used in this study and 4 dietary treatments combining lowly and highly degradable fiber (timothy hay and beet pulp, respectively) and protein (heat-treated soybean meal and soybean meal, respectively) were tested. Results indicated that fluid volume of the rumen and the rate of passage were influenced by protein, but not fiber, source. Higher rumen volumes and lower passage rates were associated with heat-treated soybean meals. The effect of dietary treatments on VFA absorption dynamics was prominent compared to the minimal changes in production dynamics. Overall, heat-treated soybean meal appears to influence VFA disappearance resulting in low concentrations within the rumen, but greater flux of VFA disappearance. In conclusion, this method demonstrated the capacity to estimate VFA dynamics beyond concentrations and molar proportions while being cost effective and more physiologically relevant. In a fourth study, we sought to investigate the growth performance and rumen VFA profile in response to different planes of nutrients and naturally occurring coccidiosis. Coccidiosis infection altered rumen isobutyrate concentrations and tended to alter major VFA concentrations suggesting the need of future work to explore coccidiosis effects on rumen fermentation. The first two investigations highlighted the potential and strength of leveraging alternative analytical tools to complement statistical approaches generally used in ruminant nutrition while concurrently improving ability to explain complex associations in the rumen. The third and fourth projects characterized the rumen VFA dynamics and profile in response to the different nutrient degradability and health status, respectively. Collectively, these investigations contribute to better understanding of rumen dynamics through novel analytical and experimental approaches.

Key words: Coccidiosis, Bayesian network analysis, Network analysis, Recursive Feature Elimination, Volatile fatty acid

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GENERAL AUDIENCE ABSTRACT

With increasing global population, income, urbanization, and changes in dietary habits, the demand for meat and milk continues to grow. The ruminant animal industries (beef cattle, dairy cattle, sheep, goat, and buffalo) carry the burden of increasing production utilizing limited resources while minimizing the negative environmental impact caused by ruminant operations. To achieve this goal the productivity of the animal must be increased, and in order to increase the efficiency of production a better understanding of factors driving the production is critical. Ruminant animals have the unique ability to convert plant fiber into human edible milk and meat through a process that predominantly occurs in the special gut compartment called the rumen. In this process several compounds are produced, and among those volatile fatty acid (VFA) is of utmost importance because it fulfills energy demands for growth, production and reproduction. The rumen is a complex ecosystem consisting of numerous variables and associations. Understanding those relationships is crucial to manipulate rumen mechanisms. The overall objective of this work was to evaluate the potential of alternative statistical approaches, which demonstrated success in other disciplines, for better depiction of complex associations and characterization of production and absorption mechanisms of rumen VFA in response to different nutrient supplies. The objective of first investigation was to evaluate a feature selection method (recursive feature elimination; RFE) and a network approach (Additive Bayesian network;

ABN) concurrently with a standard variable selection method (mixed model meta-analysis) commonly used to develop animal nutrition models. We attempted to find out the most important dietary, rumen, and animal variables for milk yield, milk fat and protein content as an example. Results indicate that the network approach was well aligned with the standard tool and can be used as a complementary approach. In our second investigation, we leveraged two networking analyses, a frequentist network which was unidirectional and a Bayesian network which was directional to explore rumen level interactions. The unidirectional network approach highlighted the most important variables in the rumen and numerous relationships among these variables. The directional network was more useful in understanding of causal associations within the system. In the third experiment we estimated the production and absorption of VFA in response to the different protein (heat-treated and regular soybean meal) and fiber (timothy hay and beet pulp) sources. The results revealed that the production of VFA was minimally affected by the diet, but the absorption was higher with heat-treated soybean meal. Our last project investigated the effect of parasitic infection, i.e., coccidiosis, and high and low levels of nutrition on growth and rumen VFA of growing lambs. Infection of coccidiosis altered a minor VFA (isobutyrate) and tended to alter total and major VFA (acetate and propionate). All these findings help to improve our understanding of rumen fermentation and subsequently develop strategies to manipulate rumen fermentation to enhance efficiency and productivity.

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Chapter 1 : INTRODUCTION

The demand for animal based products is increasing with the increasing global population, which is estimated to reach more than 9.6 billion people by 2050 (DeSA, 2015). Along the same timescale, increases in income and rapid urbanization are also occurring, both of which are expected to contribute to elevated global demand for animal-sourced foods (ASF) (Bodirsky et al., 2015; Hawkes et al., 2017; Abu Hatab et al., 2019). To meet this elevated demand, the annual production of milk, meat, and eggs is expected to reach approximately 900, 400 and 100 million tonnes, respectively, by 2030 (Pulina et al., 2017). To ensure food security by 2050, global milk production is expected to reach 1077 million tonnes (increasing from 664 million tonnes in 2006). Meat production is likewise expected to reach 455 million tonnes, upfrom 258 million tonnes in 2006 (Alexandratos and Bruinsma, 2012). Notably the demand for ASF will not increase in a uniform manner globally. Rather, it is expected to be double in African, Asian, and other developing regions, which will also see the greatest increase in population (Thornton, 2010). Given this context, ruminant animals are a pillar of global food supply given their ability to convert low quality, human inedible roughage materials into human edible food (Eisler et al., 2014). More importantly, ASF are crucial given their ability to provide highly digestible essential amino acids (lysine and methionine) which are often limited in plant-based proteins and essential fatty acids (i.e., EPA/DHA, linolenic and linoleic acid) (White and Gleason, 2022).

Although the ruminant industry plays a vital role in global nutrient supply, the sustainability of ruminant animal production, and of livestock production more generally, is in question. Often livestock sustainability is discussed with emphasis on subjects such as environmental impact, resource use, animal welfare, and the production of safe and affordable food (Capper, 2020). The role of the livestock industry in climate change has been a controversial topic for decades owing to different perspectives among the scientific community arising from common misinterpretation and variation in numbers. However, as acknowledged by several assessments, the contribution of global livestock sector to total anthropogenic greenhouse gas (GHG) emissions is 14.5% and enteric fermentation of ruminants accounts for approximately 81% (Gerber et al., 2013). Impact of livestock on climate change occurs through land use, animal feed production, feed processing, animal manure, transportation (e.g., live animals, animal feeds) and biodiversity destruction (Bellarby et al., 2013; Rojas-Downing et al., 2017). Contribution of livestock sourced carbon dioxide (CO_2), nitrous oxide (N_2O), and methane (CH_4) to anthropogenic GHG is 5, 53 and 44% respectively (Gerber et al., 2013). Within the ruminant share of GHG emissions, cattle meat and milk accounts for the most with 35 and 30% respectively, followed by buffalo (8.7%) and small ruminants (6.7%) (Opio et al., 2013). Excess loss of nutrients causing lower feed efficiency resulting in low productivity of livestock operations is the main reason behind high emissions of GHG (Gerber et al., 2013). In summary, direct, indirect, short-term and long-term strategies are critically needed to decrease global ruminant GHG emissions,.

Land and water are the basis of all food production enterprises, including livestock production. In particular, grazing ruminants occupy considerable land areas worldwide, accounting for 1/3 of arable land surface area (Herrero et al., 2013). Moreover, deteriorating reserves of nonrenewable resources (e.g., fossil fuels) and the extreme pressure on renewable resources such as land and water intensify the challenge of increasing resource use efficiency. In particular, as the population expands, it is predicted that the available land per person will decline from 0.42 hectares in 1960 to 0.19 hectares in 2050 (Silva, 2018). As available resources are increasingly limited, there is a critical need to increase animal production efficiency to meet the increasing demand for ASF (Opio et al., 2013). In addition to the challenges associated with environment and limitation of resources, concerns on animal welfare (Lovarelli et al., 2020), antimicrobial resistance (Clifford et al., 2018), and the provision of safe and affordable food for humans are other main concerns around sustainability of livestock production. Overall, it is certain that livestock sustainability counts on improving production efficiency to produce sufficient meat and milk; however, the industry must also commit to environmental accountability and maintain a positive consumer perception of animal agriculture.

Over the decades, global and US livestock industries have made considerable progress in productivity and efficiency, resulting in reductions in both resource use and GHG emissions. According to Capper (2011), U.S. beef production used 19% less feed, 33% less land, and 12% less water and reduced 16% of GHG in 2007 compared with equivalent beef production in 1977. Similar trends were observed in the Canadian beef industry in 2011 (compared to 1981) by using less land and achieving a 14% reduction in total GHG emissions to produce 1 kg of beef (Legesse et al., 2015). The improvement in production efficiency is also evident in the US swine industry, with a 29% increase in marketed pigs, largely attributed to improved breeding strategies and reductions in overall resource utilization (Cady et al., 2013). As a result of increased productivity in the US poultry industry, resource use efficiency has increased and GHG emissions per ton of eggs has decreased from 1960 to 2010 (Pelletier et al., 2014). The US dairy industry represents perhaps the most extreme example. Milk yield per dairy cow increased by 4-fold from 1944 to 2007, with simultaneous decreases in use of feed (77%), land (90%), and water (65%). This period of time also saw a 63% reduction in GHG emissions per kilogram of milk (Capper et al., 2009). This trend has continued over the past few decades, with further decreases of land (20.8%), water (down 30.5%), and fuel (down 20.2%) use per kilogram of milk from 2007 to 2017 (Capper and Cady, 2020). In summary, across all major livestock industries, emphasis on improving productivity and efficiency over the past several decades has also supported improved environmental impacts.

Historically, much of the advancement in efficiency has been achieved through genetic selection (Thornton, 2010; VandeHaar et al., 2016; Li et al., 2019). Advances in feeding management have not necessarily supported novel ways to operationalize the animal's physiology so much as they have ensured adequate nutrient supply to support genetic merit. As we work to continue to try to enhance efficiency of ruminant production systems, and to support the role of ruminants as recyclers of human-inedible materials, there is a need to identify alternative ways to leverage the ruminant animal's physiology to support its role in a sustainable and secure food supply.

Rumen fermentation is a collective outcome of all the physical, biological, and chemical parameters within the rumen, and of the complex inter-relationships among those parameters which make up the rumen ecosystem (McAllister and Newbold, 2008; Mackie et al., 2013; Owens and Basalan, 2016). Historically, scientists have taken a reductionist approach to exploring relationships within the rumen environment. Taking a reductionist lens to a complex ecosystem of relationships may result in mischaracterized understanding

(Kaput et al., 2017; Fardet and Rock, 2018), ultimately limiting our ability to formulate innovative rations to support the productivity of ruminants. To better illustrate the relationships inherent in interconnected and complex ecosystems, it is essential to utilize correct analytical tools (Barile et al., 2016; Gosak et al., 2018; Hatleberg and Hinman, 2021), and to ensure accurate quantification of important rumen parameters. Failing to do so may lead to misinterpretation of rumen mechanisms leading to less efficient attempts to modulate rumen and dietary productivity. Employing mathematical modelling to describe rumen mechanisms, including nutrient digestion and substrate fermentation has been the general approach for decades (Baldwin, 1995; France and Kebreab, 2008; Kebreab et al., 2009) with a successive development from empirical models to more precise and knowledge-driven, dynamic, and mechanistic models (Kebreab et al., 2009; Bannink et al., 2016; Tedeschi, 2019).

Empirical models are based on classical regression approaches and provide a sophisticated statistical method to estimate quantitative associations among two or more variables of interest (Tedeschi, 2019). The approach also comes with drawbacks such as limited applicability beyond the data used for the derivation, poor capacity to handle collinearity, dependence on researcher-selected variables and relationships, and limited ability to address mechanistic quantitative associations within data (St-Pierre, 2001). Meta-regression analysis is a good example for use of empirical modelling in animal nutrition and the number of articles on meta-analysis showed an exponential growth in recent years (Fontelo and Liu, 2018; Chen et al., 2021). This approach is now the standard for developing critical tools such as the National Academies of Science, Engineering, and Medicine Nutrient Requirement Series (National Academies of Sciences, 2021). Despite the advantages these modelling approaches offer, due to the weaknesses in these approaches, understanding of animal nutrition driven through these modeling exercises may be limited by data and methodological constraints. On the other hand, mechanistic models offer solutions for some of the drawbacks of empirical models, given their capacity to better depict underlying metabolic processes which is thought to produce more reliable prediction outside the calibration domain (Hanigan and Daley, 2020; Tedeschi, 2022). However, these traditional modelling approaches rely on accurate expert construction to depict the quantitative associations among variables, and often incompletely account for variable variance and measurement errors (Baldwin, 1995; Reed et al., 2015). Hence, investigating complementary statistical analytical approaches for traditional analysis can significantly contribute to our understanding of rumen fermentation mechanisms and ruminant nutrition, most notably when these approaches address some of the known shortcomings of empirical and mechanistic models.

With the advancement of affordable computing power, several novel statistical tools are becoming popular to more holistically explore the possible quantitative associations among variables within complex and correlated datasets (Morota et al., 2018; Tedeschi, 2022), such as those related to study of rumen fermentation mechanisms and ruminant nutrition. Machine learning (ML) is a sub category of artificial intelligence and is a powerful tool resulting from advancement of integration of big data concepts and high-performance computing (Liakos et al., 2018; Morota et al., 2018). Among ML approaches, Bayesian network analysis has been successfully applied in complex ecosystem studies. Frequentist network analysis is not typically considered as a ML approach, although some ML approaches can be used in conjunction with frequentist networks. Frequentist network analysis has been a popular analytical tool in social network analysis (Tabassum et al., 2018), psychological studies (Borsboom et al., 2021) and in other fields with complex, large, and interrelated datatypes. Bayesian network analysis offers advantages over frequentist approaches because of its ability to imply causation of complex quantitative associations. Bayesian Learning Networks have been successfully used to estimate inference of gene regulatory networks from RNA-Seq time series data (Behdani et al., 2019), to explore disease risks in veterinary epidemiology (Kratzer et al., 2020), and for other related applications. Network analysis has gained popularity given its capacity to explore statistical and structural properties of a set of interrelated variables (Barberán et al., 2012) and to account for multicollinearity among variables (Kim et al., 2020; P Obite et al., 2020) in a robust and holistic manner. The Bayesian approach is further strengthened by its unique capacity to handle missing data (Heckerman, 2013) through depicting incomplete knowledge about the biological system.

Another ML approach that has the capacity to capture multicollinearity among high-dimensional data is Random Forest Recursive Feature Elimination (RFE) (Darst et al., 2018). It calculates the feature importance statistics, and drops the variable with the lowest importance index, and re-derives the regression. However, use of these analytical tools in animal nutrition, specifically to understand rumen mechanisms has been limited. We suppose that using these alternative analytical tools to portray rumen fermentation mechanisms will be useful to address interrelationships, complex associations, and multicollinearity characteristics which are inherent to rumen fermentation variables, and this will help broaden our understanding on rumen fermentation.

In conjunction with adopting better analytical tools, developing methods to quantify or estimate important rumen variables is also important for advancing our ability to formulate novel diets for ruminants. Among rumen fermentation variables, volatile fatty acids (VFA) are acknowledged as the most important fermentation end product, providing approximately 70 % of the total energy requirement to fuel productive functions of the host animal (Bergman et al., 1965; Bergman, 1990). The type of the substrate fermented, microbial population, and chemical and physical status of the rumen environment influence the type and quantity of VFA synthesized in the rumen (Bannink et al., 2008), and different VFA are used with differing physiological efficiencies for various metabolic processes.

Traditionally, concentrations of rumen VFA have been used to make inferences about rumen fermentation conditions. Unfortunately, VFA concentrations are not always reliable estimators because they can be influenced by variables such as rumen volume and rumen passage rate. Further, VFA concentrations do not represent synthesis, interconversion, or absorption of VFA., (Hall et al., 2015). Isotope dilution approaches under assumed steady-state conditions and data interpretation adopting a single pool (total VFA) (Weller et al., 1967) scheme or a three-pool scheme (for major VFA) (Bergman et al., 1965) is considered to be the most accurate method of estimating VFA synthesis, interconversion and absorption (Makkar, 2008; Warner et al., 2014). However, these studies are expensive, labor-intensive, and rely on feeding strategies to induce a metabolic steady state which may not reflect normal physiological status (Hegarty and Nolan, 2007). If we can develop an affordable, less labor and time consuming experimental approach under non-steady state metabolic conditions to reflect more practical feeding situations and estimate important VFA dynamics it will immensely benefit future ruminant nutrition experiments.

To address the aforementioned challenges in estimating VFA dynamics, we suggest that evaluating time-series fermentation indicators under non-steady state (regular meal-feeding) of animals may provide an inexpensive efficient screening approach to understand fermentation dynamics. We assume that the meal serves as the substrate influx which drives the net appearance of VFA. An exponential decay curve represents the appearance of VFA and declines as available substrate decreases. Similarly, the summation of absorption and interconversion accounts for the net apparent disappearance of VFA. Overall, we assume that this method will allow us to derive rates from fermentation curves and make more representative inferences about the biological relevance of the rates estimated. Because the type of substrate is expected to influence the VFA profile we incorporate protein and fiber sources differing in rumen degradability to induce experimental variation.

To summarize, rumen fermentation variables are highly correlated and existing statistical approaches lag in aspects of capturing these highly correlated associations in a holistic manner. Further, the most accurate method to estimate important fermentation dynamics of VFA (i.e., isotope based assessments) is expensive, labor and time consuming, and assumes steady-state conditions which may not represent the normal physiological status of animals under general feeding regiments. The broad objective of this work is to attempt to address these shortcomings by deploying novel analytical tools and developing a new experimental approach. To achieve the broad objective the following secondary objectives were set.

I. To identify and evaluate alternative analytical tools to complement meta-analysis in animal nutrition.
II. To deploy promising alternative analytical tools identified from the 1st objective to better illustrate rumen fermentation variables from continuous culture experiments.
III. Develop a low-cost screening method to estimate rates of VFA net appearance and disappearance in response to the different rumen degradability of nutrients.

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Chapter 2 : LITERATURE REVIEW

IMPORTANCE OF RUMINANT LIVESTOCK INDUSTRIES

The ruminant livestock industries, which include cattle, sheep, goat and buffalo, play a significant role in global food security and nutrition, and provide livelihoods for farmers. Globally, ruminants account for largest use of land resources, approximately 1/3 of earth’s surface area, through grazing activities and harvested forage production (Herrero et al., 2013). Ruminants are an integral part of food systems and provide nutrient dense food products, such as meat and milk; however, they also contribute additional agricultural products such as hides, wool, heat, energy, and animal-powered mechanization. Products from the ruminant industries, such as meat and milk, are rich in vital nutrients and are important sources of calories, high-quality proteins, vitamins (e.g., A, B12, and B2), and minerals (e.g., calcium, iron and zinc) (Mottet et al., 2017). According to FAO (2018), 1/3 of global protein intake and 17% of calories come from animal sources, with a large portion of those coming specifically from ruminant-sourced products. Inadequate intake of animal proteins is linked to malnutrition and stunting, subsequently resulting in serious health issues including anemia and risks to pregnancy from lack of B12 (Adesogan et al., 2020). Consequences of stunting are not only linked to the increased risk of infant mortality, but also include reduced IQ score, and decreased lifetime earning potential (Victora et al., 2021). Overall, it is estimated that by the year 2030, the annual production of milk, meat, and eggs will reach approximately 900 billion, 400 million, and 100 million tons, respectively (Pulina et al., 2017). The pressure from the increasing global population, set to reach 8.5 billion in 2030 and 9.7 billion by 2050, along with increased demand for animal products due to improving economic status of developing nations, collectively suggests disproportional gains in demand for animal-sourced foods (United Nations Population Division, 2019). Even within the next 6 years (i.e., to 2029), total beef production is estimated to increase by 9% and total milk production is expected to increase by 20% (OECD/FAO, 2020). This increase in production is expected to occur concurrent with global cattle herds reaching approximately 1.8 billion by 2029 from 1.6 billion at present (OECD, 2021). Considering these global dynamics, ruminant livestock are extremely important not only because of their potential to produce high quantity of animal protein, but also for their ability to convert human inedible fibrous material to high quality nutrients (Eisler et al. 2014). However, the expansion of animal numbers is the root of a broader global challenge, namely, ruminants are notable contributors to anthropogenic climate change and resource use.

Despite all the positive contributions of ruminant livestock, there is considerable concern about the role of these animals globally and their impact on GHG emissions (Gerber, 2013), water and land use, water and air quality, and biodiversity (Steinfeld et al., 2006). In terms of CO_2 equivalents, the contribution of livestock to global anthropogenic GHG emission is 14.5% (Eisler et al., 2014). Although numbers for U.S. agriculture are lower (EPA, 2023), the ruminant industries both domestically and abroad have collectively highlighted the need for reducing emissions through pledges to achieve carbon or warming neutrality by 2040 to 2050 (Salvia et al., 2021; Nieto, 2022). Much of the focus on efforts to achieve these goals focuses on reducing enteric methane emissions. Enteric methane is the main contributor to GHG emissions from ruminant systems and is a by-product of the fermentation process through which plant materials are degraded by microbes in the rumen (Cassandro et al., 2013). Methane is also unique in that it is a short-lived GHG, meaning that mitigating methane emissions results in a direct and immediate reduction in warming potential, which is not the case for longer-lived pollutants like CO_2 (Costa et al., 2021; Ocko et al., 2021). As a result, a large body of recent literature has focused on strategies to and impacts of reducing enteric methane emissions from ruminants in the U.S. and globally (Pickering et al., 2015; Vijn et al., 2020; Arndt et al., 2021; Black et al., 2021). Beyond enteric methane emissions, ruminant animal manure also contributes to the emission of methane and nitrous oxide (Dalla Riva et al., 2014). Manure management strategies are well-acknowledged to result in trade-offs between methane and nitrous oxide emissions (Hou et al., 2015; Eilerman et al., 2016), and targeted feeding of protein sources to cattle has been proposed as a strategy to limit nitrous oxide emissions (Lee et al., 2012; Gerber et al., 2013). The need for farm-system strategies to simultaneously address multiple emission sources, and to consider broader environmental factors such as land use and water use, along with the pressures exerted by the growing demand for animal-based food have led to calls for sustainable intensification of ruminant systems across the globe (Tedeschi et al., 2015b; Dumont et al., 2018; Bateki et al., 2019).

Sustainable intensification is defined as the process where agricultural yields are increased without adverse environmental impacts and without land-use changes (Pretty and Bharucha, 2014). Despite this common proposed goal for global agricultural systems, the considerable variability in livestock productivity across different regions and production systems suggests there may be variability in relevant strategies which could be implemented to achieve sustainable intensification. Globally, ruminants are raised in 3 general styles of production system: extensive grazing on pasture; mixed systems (grazing and supplementary feed); and intensive systems (e.g., feedlot operations). Even within these categories, production and management systems of ruminants vary considerably in terms of number of animals, stocking densities, technology, capital, and labor intensity. Extensive and mixed grazing systems are pre-dominant globally, accounting for 87 to 94% of worldwide beef production (Mottet et al., 2017). According to a different source, mixed crop livestock systems (supplementary grain, hay and silage) is responsible for 70% of meat and milk from ruminants globally (Herrero et al., 2013). In US ruminant production systems, concentrated animal feeding operations and smaller confinement facilities are more prominent. These systems leverage high animal density and rely on considerable use of concentrated feed, with the aim of minimizing the time required to reach slaughter weight (Tarawali et al., 2019). Although common in the United States, feedlot systems account for only 7 to 13% of beef production globally (Mottet et al., 2017). The diversity of feeding systems globally, along with the differences in genetics, management, and environments generates a tremendous gap between actual and potential productivity in different regions globally. For example, in 2015, average milk production in North America was 9.9 tonnes per animal per year, 4.7 tonnes in Oceania, 1.9 tonnes in Central and South America, 1.4 tonnes in South Asia, and 0.5 tonnes in Sub-Saharan Africa (Bizzarri and Gapon, 2019). It is well-studied that in highly productive systems GHG emissions per unit of product are much lower (Havlík et al., 2014). As such, reducing the productivity gap in systems globally is a critical sustainability objective.

Although other factors contribute considerably to global productivity gaps, feed efficiency of ruminant animals is one factor which is known to vary both globally (Herrero et al., 2013) and within the United States (Myer et al., 2015; VandeHaar et al., 2016). Furthermore, the feed efficiency of ruminants is much lower than for monogastric animals (Cheng et al., 2022). Although feed efficiencies tend to be closer when explored on a human-edible basis (Wilkinson, 2011), the global and domestic variability in this metric of productivity suggests it may be a logical focal point for efforts to enhance sustainability of ruminant production systems. This is further underpinned by the fact that rumen fermentation is responsible for 70% of the energy available to the animal (Bergman, 1990), and for the majority of methane emissions produced from ruminant production systems (Haque, 2018; Tseten et al., 2022). Exploring strategies to optimize fermentation to achieve improved substrate use efficiency has widely been advocated as a strategy to improve the role of ruminant animals in sustainable food systems (Schader et al., 2015; Salter, 2017); however, it will require more in-depth understanding of factors controlling fermentation within the rumen environment (Bannink et al., 2016; Ungerfeld, 2020).

RUMEN DIGESTION

Physiology of Rumen Digestion

Unique structural and physiological adaptations of the ruminant digestive system allow ruminants to digest high roughage feedstuffs efficiently. The major fermentation component of the ruminant digestive system is composed of four compartments: the rumen, reticulum, omasum, and abomasum (Krehbiel, 2014). The rumen and reticulum are attached by the reticulo-rumen fold, and in practice these sections collectively create a large fermentation vat which provides a niche for numerous microorganisms, including bacteria, fungi, archea, and protozoa (Huws et al., 2018). Structurally, the luminal surface of the rumen is lined with numerous finger-like projections, papillae, which support the absorptive functions of the organ. Similarly, the luminal papillary surface of the reticulum is arranged in a unique honeycomb appearance which helps to trap large feed particles and retain them for more thorough digestion (Krehbiel, 2014). Collectively, the reticulorumen is the main site of both microbial fermentation and VFA absorption. The reticulorumen also participates in mechanical breakdown and mixing of ingested feed via coordinated smooth muscle contractions. Rumination is the process by which ruminant animals regurgitate partially fermented feed boluses for re-mastication, re-insalivation, and subsequent deglutition. The propulsive movements of the rumen and the rumination process also facilitate removal of fermentation gas by eructation during regurgitation. Following fermentation in the reticulo-rumen, the digesta enters the omasum. The omasum contains longitudinal folds thought to be responsible for absorption of water, ammonia, and VFA and for preventing large feed particles from leaving the reticulo-rumen. The last compartment of the ruminant stomach is the abomasum, which resembles the true stomach of the monogastric. The abomasum in ruminants secretes digestive acids and enzymes critical for the denaturing and preliminary digestion of proteins.

Digestion in Ruminants

Digestion in ruminants is regulated by two processes: 1) chemical breakdown of nutrients; and 2) the rate of passage of fluid and solids through the gastrointestinal tract. The two are tightly linked, with longer rates of passage being associated with greater extent of digestion due to more thorough breakdown of nutrients (Colucci et al., 1982). Similarly, a higher rate of passage or a lower rate of nutrient breakdown would provide less time for digestion hence decreasing the rumen degradability and vice versa (Dijkstra et al., 2005). Fermentative digestion, i.e., chemical breakdown of feedstuffs by rumen microbes, takes place in the rumen and represents the main site of digestion in ruminants. To better account for the role of passage rate and nutrient breakdown, scientists have developed mathematical explanations in the form of models to estimate or predict or explain the rumen degradability. A multi-step process using compartmental models used to explain the concept of digestion and rate of passage (Blaxter et al., 1956; Waldo et al., 1972; France et al., 1982; Baldwin et al., 1987b; Baldwin, 1995). The concept of compartmentalization makes sense as feed particles do not digest or pass through the digestive tract in the same rate (Sutherland, 1988). Therefore, to develop precise and accurate models, it is essential to have a correct understanding of the passage.

Rate of Passage and Extent of Degradation

Passage of fluid from the rumen to latter parts of the gastrointestinal tract (i.e., small and large intestines) in the ruminant animal is a combined process of selective retention, mixing, segregation and escape of particles and liquid (Mertens, 1993). Digestion process in the reticulorumen and latter parts of the tract are mechanistically different because the reticulorumen acts as a continuous flow fermentation vat, while the small and large intestine act more like a plugged-flow reactor (Penry and Jumars, 1987). Rate and extent of digestion is highly dependent on the physical form of the feed (Poppi, 1997). Feed physical form includes both state-of-matter (i.e., solid vs liquid), and particle size. Particles which are too large are often trapped in the reticulorumen for a disproportionate amount of time when compared to smaller, more fluid soluble particles. In a formalization of these particle size differences, nutritional models like Cornell Net Carbohydrate and Protein System (CNCPS) and the NRC treat nutrients within the rumen as falling within the category of those that are rapidly digested, slowly digested, or are indigestible. In situ degradability studies are commonly used to explore the proportions of a nutrient provided by a feedstuff or diet that fall into these different categories (Ørskov and McDonald, 1979; Ørskov, 2000). Orskov and McDonald (1979) describe the degradability/ disappearance of nutrients using an exponential equation, $p = a + b\ (l — e^{\sim ct})$ where p is the nutrient disappearance (%), a is the rapidly degradable fraction, b is the potentially degradable fraction, c is the rate of degradation of b, e is the natural logarithm, and t is time in hours.

Existing models of rumen function illustrate digestion as a mass action function of available substrate and described as a first-order process with respect to substrate (Waldo et al., 1972; Mertens and Ely, 1979). Development of the mathematical expression of a first order digestion model is fairly simple compared to its biological conceptualization. Four types of first order models are commonly identified, including: 1) single compartment, single reaction, 2) single compartment, multiple simultaneous reactions, 3) multiple compartments, single simultaneous reactions, and 4) multiple compartments, single sequential reactions (Mertens, 1993). The first order models assume that rate and extent of digestion are limited only by intrinsic properties of the substrate alone. However, contemporary biological understanding suggests that there are likely extrinsic factors, such as microbial mass or enzymatic activity, that potentially limit the rate of reaction (France et al., 1982; Baldwin et al., 1987a). Models developed to consider these factors are termed second order models. Henri–Michaelis–Menten (HMM) kinetics is a common formalization developed for exploring enzyme-driven reactions, and is used as the basis for a more complex digestion model, which considers a reversible, four-compartment system with both first- and second-order reactions (Segel, 1975).

Although first order and second order reaction kinetics have historically been used to explore rates of nutrient breakdown, systems which concurrently consider passage and degradation are needed to fully explore the impact of each factor independently. More comprehensive systems for ration formulation such as the CNCPS, and the National Academies of Science, Engineering, and Medicine models for dairy and beef ration formulation both rely on a formalization of digestion which considers both rates of passage and rates of degradation. Since feed evaluation systems have changed more towards mechanistic models from empirical models, fractional degradation rates of the particular nutrient (Kd) and the rate of passage (Kp; %/h) were included in equations (Hvelplund et al., 2009). Different techniques, including in situ techniques and in vitro methods (in vitro gas measurements and rumen evacuation technique) have been used to measure Kd of a particular nutrient in the rumen (Huhtanen and Sveinbjörnsson, 2006). Generally, Kp is determined using fluid flow markers and the first-order kinetics considering a single compartment. However, Kp can also be calculated using equations developed by the NRC (2001). By combining Kd and Kp the extent of degradation of the nutrient in the rumen can be estimated with a high accuracy (France et al., 1990; France et al., 1993). Use of different fractions of protein (A, B, and C) and Kd to estimate RDP and RUP and use of calculated Kp in protein models (NRC, 2001) can be given as an example. In these models, predictions of RDP flow are a function of Kp, Kd, and A and B protein fractions.

Degradability of Fiber

As forage accounts for 40 to 100% of the ration of ruminants (Peyraud et al., 1996; Bargo et al., 2002) fiber is the main nutrient fermented, and a critical source of energy for maintaining animal productivity and health. Plant fiber cannot be digested using mammalian enzymes, which is partially why ruminant animals have co-evolved with an ecosystem of microbes to support fermentation processes. In addition to supporting a critical energy source for ruminant animals, fiber consumption is associated with chewing and salivation, rumination, gut motility, and overall rumen health (Zebeli et al., 2012; Beauchemin, 2018; Natnael et al., 2020).

Fiber in forages is composed of structural polysaccharides including cellulose, hemicellulose, lignin, and pectin (Ribeiro et al., 2016). Neutral detergent fiber (NDF) is the most common measure of structural fiber in ruminant diets, and typically accounts for 30 to 80 % of forage organic matter (Buxton and Redfearn, 1997). Collectively lignin, cellulose and hemicellulose make up NDF, and as plant maturity increases, so too does NDF percentage (Ball et al., 2001). As a result, NDF percent has been advocated as an indicator of intake level in ruminants (Beauchemin, 1996; Oba and Allen, 1999). Acid detergent fiber (ADF), the other major fiber component in forage, is comprised of cellulose and lignin only (Jung, 1997). Neither mammalian nor rumen microbial organisms have measurable lignin degradation capacity, therefore lignin in forages is a primary indicator of digestibility.

Fiber (cellulose and hemicellulose) degradation occurs in the rumen (Huhtanen et al., 2006) in the range of 30-50%, and is dependent on the type and length of forage. Longer forage particles are subject to improved degradation within the rumen when compared with processed, shorter forage particles because longer particles resist fluid outflow and are retained in the rumen for an extended period of time (Gregorini et al., 2015). However, shorter forage particles are more accessible to microbial infiltration and degradation (Saylor et al., 2020), thus those short particles which are retained within the rumen long enough for fermentation are highly digestible. Again, these interplays between passage rate and nutrient breakdown create complex dynamics which shift expectations about dietary or feedstuff fiber degradability.

Degradability of cellulose and hemicellulose also depend on the level of lignification of the forage (Jung and Deetz, 1993). Lignin is considered as the major constraint of fiber digestibility, because lignification of plant cells occurring as a part of plant maturation ties up otherwise degradable nutrients. In addition to lignin infiltration, 4 major factors control ruminant fiber digestion: 1) plant structure and composition, regulates bacterial access to nutrients; 2) population densities of the predominant fiber-digesting microorganisms, influencing potential degradation capacity of the ecosystem; 3) microbial factors that control adhesion and hydrolysis, influencing the efficacy of fiber degrading populations; and 4) animal factors that increase the availability of nutrients through mastication, salivation and digesta kinetics (Cheng et al., 1991). Each of these factors has some degree of genetic control, and each has some elements which are influenceable by management or diet. For example, the microbial community of fiber degrading microorganisms is highly individual among animals (Henderson et al., 2015), partially due to genetics of the individual and inoculation by their dam at birth (Cheng et al., 1991). Microbial communities can be influenced by diet (Jami and Mizrahi, 2012; Henderson et al., 2015; Li et al., 2021), for example, diets supporting a decline in rumen pH are not conductive for most fiber degrading organisms (Li et al., 2021). Due to this complexity of factors controlling the fiber degradation, there is still unclear quantitative roles for these varying factors and how they influence degradation.

The mechanical processes for cellulose and hemicellulose breakdown are multi-step and often rely on suites of microorganisms. Three classes of microbial enzymes including endo-β-1,4-glucanase, cellobiohydrolase, and β-glucosidase are critical for cellulose breakdown (Dimarogona et al., 2012; Lakhundi et al., 2015). The variable structure in hemicellulose makes the degradation more complex than cellulose, and it requires enzymes capable of depolymerizing the hemicellulose backbone including endoxylanase , β-xylosidase , and glucuronoxylan hydrolases (Houfani et al., 2020). In addition to the enzymes involved in degradation of major fiber components, several other enzymes such as α-L-arabinofuranosidases, acetyl xylan esterases, feruloyl esterases, and α-glucuronidases are also essential in plant cell wall degradation as they act to remove the side chains on the xylan backbone. Coordinated interaction of all these enzymes is required for efficient digestion of fiber within the rumen, and the capacity of microorganisms to produce these enzymes varies. As a result, coordinated teams of microorganisms are thought to be necessary to facilitate optimal fiber degradation within the rumen ecosystem. Despite this preliminary understanding, we have limited incorporation of this level of complexity in representing ruminal fiber degradation into our tools and techniques used for describing ruminant diets and nutrient availability.

Importance of Increasing Fiber Digestion

To enhance productivity and profitability in the ruminant livestock industry it is critically important to increase fiber digestion. Incomplete or reduced fiber digestion in ruminant animal limits intake, which results in reduced nutrient availability, impaired animal productivity, and increased manure production (Adesogan et al., 2019). Conversely, a 1-unit improvement in forage NDF digestibility in dairy cattle led to increases in DMI and milk output of 0.17 and 0.25 kg/d, respectively. (Oba and Allen, 1999). Lignin is the main component that hinders the fiber digestion and in corn cell walls, a 1 percentage increase in lignin concentration decreases cell wall degradability by 2% (Grabber, 2005) . The importance of increased fiber digestion on production is also well depicted with the example of ryegrass, where a 5 to 6% increase in fiber digestibility was reported to support a 27% increase in milk production (Smith et al., 1998).

Improving fiber digestion in ruminants is also critical to maximize the energy supply from fibrous feeds that are inedible to humans, this supported limited use of concentrate feeds that could be consumed by humans for energy and supports the ecological role of ruminants in societies as recyclers of otherwise unusable nutrients. For this reason, both forage- and non-forage-based fiber sources are critical for use in ruminant diets.

Non-forage Fiber Sources

In the agriculture industry, many crop species are processed to recover particular fractions of the plant, and in many cases, the byproducts are high in fiber. This high fiber content adds little or no market value for human or common industrial uses; however, it does make these byproducts particularly suitable as feedstuffs for ruminants (García-Rodríguez et al., 2019; Vastolo et al., 2022). There are several common agro-industrial by-products such as brewers grains, corn gluten feed, distillers grains, soybean hulls, sugar beet pulp, and wheat middlings that can be used as non-forage fiber sources (NFFS) in ruminant diets (Bradford and Mullins, 2012; Wang et al., 2018). A study reported that California alone used over 2.5 million tonnes of 9 different non-forage fiber sources in 1992, with a market value of over $230 million (Grasser et al., 1995). Utilizing NFFS in the ruminant feeding industry has several advantages in maintaining sustainability, especially in an era where sustainability of livestock industry is frequently and increasingly questioned. These advantages include an additional revenue for agro-industrial stakeholders and limited cost to dispose byproducts. These economic advantages facilitate sustainability in the ruminant industries by making NFFS an integral part of feeding regimes, helping to ensure these nutrients are captured within the food production system rather than being wasted(Habeeb et al., 2017; Mohsen et al., 2021; Vastolo et al., 2022). Calculating the economics of feeding NFFS is challenging because market values can deviate substantially due to the fluctuations in supply. From a nutritional standpoint, many NFFS provide other valuable nutrients in addition to digestible fiber, most often supplying higher levels of protein. As a result, there is considerable work within the industry to explore NFFS use to replace both cereal grains and oilseed meals in ruminant rations (Bradford and Mullins, 2012; Ertl et al., 2016; Halmemies-Beauchet-Filleau et al., 2018; Mohsen et al., 2021).

Sugar beet pulp (BP) is the byproduct of sugar extraction from the cultivated beet *Beta vulgaris* and its use to replace grains as energy source in dairy cow diets is common (Münnich et al., 2017). The US is one of the world's five largest producers of sugar beets (Habeeb et al., 2017). The north-central and western regions of the U.S. are main BP producing regions (Habeeb et al., 2017). Beet pulp is insoluble in water and the major constituents are cellulose, hemicellulose, lignin, and pectin. Beet pulp is commonly used as an animal feed and is thought to provide highly digestible fiber. Although an excellent fiber source, sugar BP is lower in many other nutrients, for example, it contains only 9.1% CP, 0.72% calcium, and 0.20% phosphorus (Habeeb et al., 2017). Dry BP product contains approximately 10% moisture and has similar energy levels to barley (Habeeb et al., 2017) .

Beet pulp contains highly digestible NDF (40.6 % of DM) (Naderi, 2014) and helps to maintain optimum rumen fermentation and encourages acetate production (Habeeb et al., 2017). Although BP is high in neutral detergent soluble fiber, i.e., pectin (23% DM) (Yapo et al., 2007), several studies have reported that BP based diets stabilized pH within a healthy range in the rumen (Mojtahedi and Mesgaran, 2011; Shahmoradi et al., 2016). This pH stabilizing potential of BP can be attributed to the low starch content. Further, although pectin ferments rapidly(Marounek et al., 1985), it is associated with less lactic acid production (Strobel and Russell, 1986) . Responses of animals (DMI, rumen fermentation and production) to BP based diets have not been consistent in previous studies (Bodas et al., 2007; Münnich et al., 2017). Some studies reported no effect of BP on DMI (Dann et al., 2007; Fanchone et al., 2013), while others reported increased (Poorkasegaran and Yansari, 2014) or decreased DMI (Alamouti et al., 2009). A meta-analysis on the BP diets in dairy cows reported the effect of BP on DMI depends on the cow’s DMI level. Further, this analysis highlighted BP had a negative impact on net energy of lactation, and no effects on rumen VFA concentrations or molar proportions. Although BP did not increase milk yield, it was found to support increased fat corrected milk yield (Münnich et al., 2017).

Degradability of Protein

Crude protein (CP) is broadly categorized into rumen degradable protein (RDP), which can be utilized by rumen microbes and rumen undegradable (RUP) or rumen bypass protein which escapes the rumen (Schwab and Broderick, 2017; Putri et al., 2021; Mi et al., 2022). Rumen degradable protein is degraded by enzymes (e.g., proteases, peptidases, and deaminases) secreted by rumen bacteria; RDP breakdown yields peptides, ammonia nitrogen and amino acids used to synthesize microbial CP (Brock et al., 1982; Wallace et al., 1997; Putri et al., 2021). Microbial CP flows as a part of the liquid and solid phases of digesta and is subsequently absorbed as amino acids and peptides in the intestine, providing 50% to 80% of the absorbable true protein (Bach et al., 2005). The remaining absorbable protein comes from protein sources which bypass the rumen. This RUP flows directly to the abomasum and small intestine, where it is digested by enzymes originating from the host animal. Approximately 80% of RUP is absorbed in the small intestine as amino acids. Compared to microbial CP, RUP is more important for providing high-quality amino acids to highly productive ruminants (Owens et al., 2014) because modulating the amino acid composition of RUP is considered a more reliable strategy to convey amino acids to the host animal than shifting amino acids available in RDP, which first are degraded and utilized by microbial metabolism before becoming available to the host.

Dietary CP in the ruminant diet supports three major functions. It helps to fulfill RDP requirements of rumen microbes for optimum digestion of carbohydrate microbial protein synthesis. Dietary CP also provides protein required for host animal maintenance, growth, health, and production. Finally dietary CP supports the amino acid requirements of highly productive ruminants (Tedeschi et al., 2015a). The ratio between RDP: RUP is critical for efficient ruminant productivity. Generally, plant based feed sources are high in RDP (Batajoo and Shaver, 1998) and proteins of animal origin are generally higher in RUP thus making more available protein for the host animal (Habib et al., 2013). The ratio of RDP and RUP also varies with the processing methods of feed sources, a great example of this variability is found in soybean meal. Soybean meal, which is processed using a heat treatment method (e.g. roasting, extruding), is considered as low in RDP hence high RUP or rumen by-pass protein. Low RDP levels can decrease ruminal NH_3-N production hence decreasing microbial CP production. Excess RDP will be broken down into NH_3-N and metabolized into urea in the liver before being eliminated in the urine (Bahrami-Yekdangi et al., 2016). As such, moderating RDP availability for microbial growth requires attention both to under- and over-feeding.

Degradation kinetics of CP within the rumen are affected by the type of feed (Batajoo and Shaver, 1998), interactions with other nutrients (ex: synchronization with carbohydrates), and the predominant microbial population (Bach et al., 2005). Protein solubility and structure are key factors determining susceptibility to microbial degradation. When proteins are composed of insoluble and slowly degraded substances (e.g., prolamins and glutelins), they are more resistant to microbial degradation. Conversely, highly soluble proteins like globulin are readily degradable rumen microbes (Romagnolo et al., 1994; Andrade-Montemayor et al., 2009). Irrespective of solubility, proteins containing disulfide bonds, like those found in albumin, are slowly degraded in the rumen. Furthermore, certain peptide bonds are more resistant to ruminal degradation than others. Slower hydrolyzation of dipeptides formed of Lys-Pro in comparison to dipeptide Lys-Ala is an example (Yang and Russell, 1992).

Ruminal factors also play a prominent role in regulating protein degradation. The rate of passage through the rumen is inversely related with protein degradation (Ørskov and McDonald, 1979). Equations such as those used in the NRC (2001) attempt to account for passage rate for a wide range of feed stuffs when estimating RDP. For example, this model estimated rate of passage and protein degradation of ryegrass silage, alfalfa hay, and soybean meal, and reported increased passage rate decreased protein degradation by 1.2, 2.1, and 3.5 percentage units, respectively (NRC, 2001). As previously explained, these models (which explicitly account both for the potential breakdown of nutrients and for the role of passage rate), have evolved out of targeted individual studies to better explore the impacts of known ecosystem factors on nutrient degradability. Despite this biological alignment, this model still returned biased assessments of rumen fermentation characteristics when compared with measured observations (White et al., 2017).

In addition to passage rate, there are a number of additional factors which are expected to influence protein degradability in the rumen. Ruminal pH ranging from 5.5 to 7.0 is considered optimal for rumen proteolytic enzyme activity (Kopecny and Wallace, 1982), and at the lower end of normal ruminal pH, protein degradation tends to reduce. Interaction of rumen pH with the available substrate was reported to dictate the predominant type of rumen microbes which has a significant impact on protein degradability (Bach et al., 2005). This situation was clearly demonstrated in a study where regular soybean meal and heat treated soybean meal were incubated either in a rumen of a dairy cow (60:40 forage to concentrate) or in a rumen of a beef cow (10:90 forage to concentrate) under same rumen pH (>6.0). The beef cow rumen and ration resulted in lower protein degradation than the dairy rumen and ration (Devant et al., 2001). Another factor affecting protein degradability is the interactions of nutrients that will lead to interactions between proteolytic enzymes with other enzymes (Bach et al., 2005). Studies have shown that addition of amylase increased protein degradation of cereal grains indicating that starch interferes with the protein degradation (Assoumani et al., 1992; Tomankova and Kopecny, 1995). When protein is bound to NDF, protein will degrade only after microbial depolymerization of cellulose (Debroas and Blanchart, 1993). This was further confirmed through the increased protein degradation from 42.4% to 53.1% after cellulases were added to an in vitro digestion (Kohn and Allen, 1995). In summary, protein degradation in the rumen requires the combination of several proteolytic and non-proteolytic enzymes.

VOLATILE FATTY ACIDS

Volatile fatty acid is one of the main end products of rumen fermentation along with gases, heat, and small amount of lactic acid (Dijkstra et al., 1993). The nutritional importance and relative proportions of individual VFA have been well-recognized since the 1940s (Bergman, 1990). Volatile fatty acids are the major form of energy supply for the ruminant animal, because VFA deliver approximately 70% of the metabolic energy required for productive functions, such as growth and milk production (Bergman, 1990). The type of VFA produced in the rumen is regulated by the substrate being utilized, microbial population, thermodynamics, and other rumen conditions like rumen pH (Bannink et al., 2008). Acetate, propionate, and butyrate are the major VFA produced, and are typically found in that order in terms of ruminal concentration or molar proportion. Although the ordering of VFA concentrations rarely changes, the relative concentrations, or molar proportions, are typically quite sensitive to the changes in the diet. These major VFA account for 95% of the total rumen VFA, and the remaining 5% are typically comprised of valerate, iso-valerate, iso-butyrate, caproate, 2-methylbutyrate and traces of higher acids. The relative production of different VFA, and the downstream supply of these VFA to the host animal, are important because each VFA is utilized through different biochemical pathways by the host. The molar ratios of acetate to propionate to butyrate in mammals vary from approximately 75:15:10 to 40:40:20 (Bergman, 1990). All major VFAs can be utilized to generate energy in the form of ATP; however, propionate specifically is unique in that it is glucogenic and constitutes the major substrate in hepatic gluconeogenesis (between 46 and 73%) in ruminants

(Herdt, 1988). Propionate is also unique in that it is critical for the synthesis of milk lactose (Wiltrout and Satter, 1972; Reynolds et al., 2003; Larsen and Kristensen, 2013). Acetate and butyrate are non-glucogenic VFA and are utilized in synthesis of milk fatty acids (Seymour et al., 2005; COZMA et al., 2013; Urrutia and Harvatine, 2017).

Synthesis of Volatile Fatty Acids

The main substrates for ruminal microbial fermentation include structural (cellulose and hemicellulose) and non-structural carbohydrates (sugar, starch and pectin), mainly from plant materials in the diet of the ruminant animal (Nagaraja, 2016). Dietary carbohydrates are degraded to hexoses and pentoses, which then produce pyruvate before being fermented to VFA. Formation of propionate occurs primary through succinate on forage-based diets and via acrylate on concentrate-based diets (Ghimire, 2015; Ungerfeld, 2020). Similarly, both acetate and butyrate are formed through pathways which rely on acetyl CoA as an intermediate (Ungerfeld, 2020). Though rumen microbes utilize carbohydrate as the main source of substrate if the diet is high with rumen degradable protein, a considerable amount of VFA can be synthesized using protein (France and Siddons, 1993). The VFA produced from lipid is generally low. Proteins are first hydrolyzed to amino acids and then deaminated before converting to VFA. Minor VFA, such as valerate and branched chain VFAs (iso-butyrate, iso-valerate and 2-methylbutyrate acids), are essential for the growth of certain bacterial species, and are derived from proline, valine, leucine and iso-leucine respectively (Cotta and Hespell, 1986a; Cotta and Hespell, 1986b).

Despite the attempts of modelling stoichiometry of fermentations using mechanistic or empirical models (Dijkstra et al., 2008), prediction of rumen VFA dynamics (synthesis, absorption and interconversion) is still not achieved satisfactory (Bannink et al., 1997b; Hanigan et al., 2006;

Noziere et al., 2010). Poor prediction of VFA profile can be due to the statistical methods, representativity of the data sets or the assumptions about efficiency of microbial synthesis, VFA removal rate from the rumen (Noziere et al., 2011). Although the biochemical pathways associated with VFA production have been characterized, estimation of supply of individual VFA for metabolism remains a challenge (Noziere et al., 2011). To better illustrate the VFA dynamics, a few aspects need to be considered. The majority of the rumen models to predict VFA have been limited to steady state conditions using constant inputs (Baldwin et al., 1987c; Bannink and De Visser, 1997; Bannink et al., 1997a). However, in reality rumen dynamics and contents fluctuate during the day (Sun and Gibbs, 2012), hence rumen models need to rely on non-linear relationships. Considering different substrates (forage vs concentrate) in a single pool without accounting for differences in composition and in fractional degradation and rumen outflow rates is another limiting factor of majority of rumen models. Moreover, the lack of understanding of the effect of different nutrient degradability on rumen VFA synthesis is noteworthy. Therefore, a rumen level approach to estimate synthesis and absorption fluxes considering above concepts are vital (Sutton et al., 2003).

Absorption of Volatile Fatty Acids

The majority of the VFAs produced in the rumen are absorbed across the rumen wall and a proportion of 10 – 20% in sheep and up to 35 % in cattle pass to the omasum and abomasum are subsequently absorbed (Dijkstra et al., 1993). Quantitatively a dairy cow absorbs approximately 100 mol of VFA per day (Storm and Kristensen, 2010). Three different absorption mechanisms of VFA through the rumen wall are suggested and those are i) passive diffusion, ii) bicarbonate dependent absorption and iii) bicarbonate independent absorption. Passive diffusion of nonionized VFA across ruminal epithelium is the most common approach and occurs according to a concentration gradient. This concentration gradient estimates transfer is highest for acetate, followed by propionate, and lowest for butyrate among major VFAs. At neutral pH, major VFA have quite similar concentration and absorption, but at low pH levels, VFA with higher number of carbons show a higher fractional absorption rate because of the greater lipid solubility (Dijkstra et al., 1993; López et al., 2003). At higher rumen pH, most of the VFA exists in the associated form, and the fractional rate of absorption is reduced. About 28 to 60% of acetate and 69 to 74% butyrate absorption occur through passive diffusion (Penner et al., 2009). The absorption through anion exchangers increases the pH in the rumen because bicarbonate and H can be converted to CO_2 and water by carbonic anhydrase (Gäbel et al., 2002). Acetate can also be absorbed through bicarbonate dependent transport, which is less lipophilic, and it can lead to increased concentrations of VFA in the rumen (Aschenbach et al., 2009). Bicarbonate independent and protein mediated transport are the other main mechanisms of VFA absorption from the rumen (Aschenbach et al., 2009; Penner et al., 2009). However, the exact percentage of major VFA absorbed from each of these mechanisms has not been well understood. Fractional absorption rates of VFA can be affected by the rumen pH. Dijkstra et al. (1994) reported no effect of ruminal on fractional absorption rate of acetic acid but increased for propionic and butyric acid when pH decreased from 7.2 to 4.5.

Higher concentration gradients between the rumen and the portal circulation can help account for the difference in the fractional absorption rates among the VFA. Because of the differences in absorption rates despite the similar acid dissociation constants, they exert different effects on ruminal pH. Collectively, these factors suggest that capacity to reduce ruminal pH is higher in acetic acidlower in propionic acid, but greater than butyric acid. The concentration gradient is also influenced by rumen movements because it increases the mixing of the rumen and subsequently increases the VFA concentration at the ruminal epithelium, likely resulting in higher absorption rates.

Dietary Effects on Volatile Fatty Acid: What we know and knowledge gap.

The type of feed source has a great impact on the composition of microbial population hence on the ratios of acetate, propionate, and butyrate generated in the rumen (Alstrup et al., 2016; Bharanidharan et al., 2018). Generally, high fiber diets encourage growth of acetate producing bacteria and the acetate, propionate, butyrate molar proportions would typically be in the range of 70:20:10 (France and Siddons, 1993; Dijkstra et al., 2008). The diets which are high in starch or concentrate feed facilitate the propionate producing bacterial species and produce more propionate at the expense of acetate. In some instances, diets rich in concentrates may encourage development of a large protozoal population leading to more production of butyrate than propionate (Williams and Coleman, 1997). Dietary forage to concentrate ratio is the most studied area of rumen fermentation and VFA profile. Sutton et al., (2003) investigated two diets with different ratios of forage to concentrate (60:40 normal diet and 90:10 low forage diet) and observed increased production rate and molar proportion of propionate in response to the high-concentrate diet compared to a normal forage based diet. The same study also reported a decreased butyrate production rate in high-concentrate diets, with no change in acetate observed. A study where cows were fed with low concentrate (8% of dietary DM) and high concentrate (64 % of dietary DM) reported decreased molar proportions of acetate by 20% in high concentrate diet and increased molar proportions of propionate, butyrate, valerate, and isovalerate by 28, 32, 39, and 53% respectively. Further high concentrate diets lowered the acetate to propionate ratio (2.36 vs. 3.98) (Penner et al., 2009). Based on these data, there is a well-established relationship between forage to concentrate ratio and VFA molar proportions.

Within the existing literature, several major drawbacks of VFA production can be identified. Limited data availability on VFA production and overall VFA dynamics ultimately results in large prediction errors in models used to predict VFA production (Ghimire et al., 2017). Lack of data availability on minor VFA is another area of concern. The cost and the difficulty associated with the more accurate VFA production method (iso-tope based method) certainly is the main reason for lack of data. Another area which needs more research includes investigating VFA production in response to a wide range of feed sources and especially different nutrient degradabilities. Gleason et al., (2022) investigated how different rumen degradability of protein and fiber influence rumen VFA production, interconversion and absorption and observed that the synthesis and absorption of butyrate increased in response to highly degradable fiber while other VFA were unaffected. The above study also investigated the effect of another dietary fiber source, timothy hay on VFA dynamics and reported minimal effects except on VFA interconversion. The concept of physically effective fiber which accounts for particle length and NDF content is another important measure of dietary fiber (Yang and Beauchemin, 2007). Previous studies on use of non-forage fiber sources in ruminant diets mostly reported VFA concentrations or molar proportions only (Mojtahedi and Mesgaran, 2011; Guo et al., 2013; Asadollahi et al., 2016). Therefore, considering physical form and rumen degradability of the fiber source is crucial when animals are fed with non-forage fiber sources such as beet pulp, citrus pulp etc. and data on VFA production accounting for these characteristics of fiber is in need.

Similar to the investigations on dietary fiber, the majority of the previous studies have reported the effect of dietary protein on rumen VFA profile as concentrations or molar proportions of VFA (Davidson et al., 2003; Oh et al., 2008). Moreover, in most cases the objective was to investigate the effect of different protein sources or different protein to energy ratios rather than different degradability of the protein (Dong et al., 2017; Shen et al., 2018). Davidson and colleagues (2003) tested 5 diets varying in crude protein and RUP and reported no differences in molar proportions of acetate, propionate or isobutyrate but significantly higher concentration and molar proportion of butyrate in cows receiving high level of crude protein and RUP. Furthermore, they reported significant differences in molar proportions of isovalerate and valerate. A study by Gleason and colleagues (2022) where experimental diets were consisted with protein sources (soybean meal and heat treated soybean meal) with different ruminal degradability did not observe many changes in VFA production or absorption, but differences were significant in interconversions. In summary, there is a major knowledge gap in VFA dynamics in response to different degradability of nutrients and the high cost and labor intensiveness in existing methods to estimate VFA dynamics needs to be addressed.

Effect of Coccidiosis, Gastrointestinal Parasitic Infection on VFA Dynamics in Ruminant

Coccidiosis is primarily a protozoan infection though taxonomically ruminant coccidia can be caused by a group of unicellular parasites from several different genera, e.g. *Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis* species, and *Eimeria* species (Bangoura and Bardsley, 2020). In ruminants *Eimeria* species are predominant and they develop in the small intestine and large intestine and affect young animals in particular. Numerous *Eimeria* species have been identified across ruminant species and previously it was understood that sheep and goats share *Eimeria* species but later it was discovered that these 2 species harbor their own panel of *Eimeria* species. In sheep 12 intestinal and 1 abomasal *Eimeria* species have been identified but the abomasal *Eimeria gilruthi* is assumed to be a stage of another intestinal *Eimeria* species rather than an individual species.

Life cycle of Eimeria species consist of 3 major phases including an exogenous phase of maturation of the oocyst (sporogony), which occurs outside the host, and two parasitic endogenous phases within the host with an asexual followed by a sexual multiplication. The endogenous phase takes about 1 to 3 weeks depending on the species and takes place after ingesting externally matured oocysts. Theoretically each oocyst ingested could be the origin of 30 million oocysts excreted in fecal matter thus making the proliferative potential in the host very high. These ingested oocysts get ruptured due to the effect of intestine enzymes, temperature, and pH, and subsequently release infectious content in the intestine. The infectious content is called sporozoites (Kowalik and Zahner, 1999) and it invades an enterocyte of the host animal and gain nutrients from the host cell through the parasitophorous vacuole (PV) (Saliba and Kirk, 2001). Inside the PV asexual replication of the parasite begins and after one cycle of replication it leaves the host cell by rupturing it. Each parasite then infects a new host epithelial cell. After the completion of asexual replication, the parasite enters the sexual replication phase and produces either a macrogamont (male stage) or a macrogamont (female stage) (Bangoura and Bardsley, 2020). Later on, after maturation, the macrogamont releases many microgametes and fertilizes the macrogamont and forms a zygote that will mature to a oocyst, which will eventually be excreted into the environment through the feces.

Coccidia causes substantial economic losses to the small ruminant industry due to the clinical disease, characterized by diarrhea, reduced feed intake, enteritis, dehydration, anorexia, and potentially death and subclinical infections which can compromise weight gain (Chartier and Paraud, 2012). Coccidiosis can become a serious problem under intensive breeding conditions and high animal density in feedlot operations. Therefore the disease should be always regarded and treated as a herd disease. Once the parasite is present in the herd, it can be spread widely within a few life cycles of the parasite. Older animals develop a relatively improved immunity compared with young animals, causing excretion of high numbers of oocysts, and further contaminating the environment. When the parasite is identified within the herd it is advised to follow herd level treatment either with direct oral administration or water treatment for 5 days or 21 days with an anti-coccidian drug (amprolium; Corid). The level of infection depends on the species of *Eimeria*, its replication ability, infection dose, immunity level of the animal and concurrent infections with other pathogens (Bangoura and Bardsley, 2020). Initial and predominant infection sites include the small intestine and large intestine, and cause direct damage by the destruction of host cells. Infection can go deeper up to the submucosal layers including lymphatic vessels (Lindsay et al., 1990) and severe inflammatory conditions can be observed due to the immunosuppression (Gregory, 2019). The studies where the effect of coccidiosis infection on rumen fermentation of sheep investigated are not common but it can be assumed that there should be an effect due to the impaired function of gut and overall metabolism that will negatively impact rumen fermentation.

Methods of Measuring VFA Dynamics

Dynamics of VFA in the rumen is an equilibrium among VFA production (appearance) or synthesis, interconversion among VFA and absorption (disappearance). In most of the previous studies, VFA are reported as concentrations or molar proportions mainly because of the challenge in measuring the actual production of VFA in the rumen. Measuring the actual production of VFA is constrained due to the dynamics in the VFA pool as well as the overall dynamics of the rumen liquid pool. Methods of measuring VFA can be broadly divided into 2 categories as tracer based methods and non-tracer based methods. Non-tracer based methods include: the VFA appearance measurements in blood collected from the portal vein (Huntington et al., 1983); in vitro rumen fermentation experiments; and the ruminal evacuation and introduction of a VFA solution into the ventral sac of the washed rumen (Dijkstra et al., 1993). The intraruminal infusion of VFA labeled with radioactive (Sutton et al., 2003) or stable isotopes (Noziere et al., 2000; Kristensen, 2001), and the continuous infusion of non-labeled VFA in a non-evacuated rumen (Peters et al., 1990) fall under the tracer based methods. Use of portal appearance of VFA is limited due to the rumen wall metabolism and the intensive nature of portal catheterization studies. The ruminal evacuation method has limitations due to the disturbance it causes in rumen microbiome and overall function. Therefore, tracer based methods provide the current state-of-the art method to evaluate rumen dynamics.

Tracer Based Methods

Tracer based methods use either radioactive isotope or stable isotope to measure VFA concentrations and results from both methods are similar (France and Dijkstra, 2005). The use of radioactive isotopes has been limited in animal experiments given associated health risks and regulatory compliance. In isotope dilution techniques, either one labeled VFA or a mixture of labeled VFA can be used. When only one labeled VFA is employed, the rate of VFA production is estimated from enrichment and the relative fraction of VFA in the samples. Total VFA is then assumed to be a single, homogenous pool (Weller et al., 1967). When separately labeled VFA are used each major VFA is considered as a different pool of VFA and interconversion among VFA are also can be estimated (Sutton et al., 2003). The first method is generally not in use due to its lack of capacity to estimate absorption rates and interconversion among VFA (Hegarty and Nolan, 2007). Therefore, the isotope dilution technique using all major VFA isotope provides the most accurate estimates of VFA synthesis, interconversion, and absorption from the rumen. Despite the great potential of stable isotope method, use of the method is limited by both the intensive technology requirement and high cost.

Non-tracer Methods

In vitro rumen fermentation, portal arterial difference, and perturbation of the steady state are a few widely used non-tracer based methods. In in -vitro fermentation experiments, ruminal contents are sampled post feeding and incubated anaerobically in an artificial rumen condition. In order to determine the VFA production rate at zero time, the concentration of VFA is measured at various time intervals and the calculated decline rate is employed (Corley and Murphy, 2004). The difference of blood VFA concentration between arterial flow and portal flow is the second approach. A critique of this method is it can underestimate the production of butyrate which extensively metabolized in ruminal epithelium (Kristensen et al., 2000). In the third approach, when the rumen reaches a steady state, an increasing level of known VFA concentration is constantly administered to reach a new steady state circumstance. The production rate (mol/d) for control treatments is indicated by the x intercept of the regression line, which shows infused VFA (mol/d) vs. VFA concentration (mM) as well as the concentration of VFA for control treatments. To experiment this Martin et al. (2001) infused propionate but the assumption that the slope or k not altered by the infusion is questionable. High propionate can lower pH and influence the rate of absorption, which could lead to biased production rate estimate. Acetate to propionate ratio can change as a result of increasing propionate concentration because higher product concentration caused by thermodynamics state, can make substrate fermentation more favorable towards another product. Inability to estimate de novo synthesis and interconversion of VFA is a major drawback of non-tracer based methods.

Typically, VFA concentrations are used as a measure of rumen fermentation and to make inferences on dietary effects. Concentration does not serve as a reliable measurement since it is influenced by external factors such as rumen fluid volume, rate of absorption and rate of passage of VFA (Hall et al., 2015). Therefore, for more precise and accurate estimations of VFA synthesis, absorption and interconversion measure of rumen pool size is critical. Development of less-invasive approaches to measure rumen liquid is challenging but ruminal liquid markers can be beneficial in achieving this.

Polyethylene Glycol (PEG) as a Fluid Marker

Polyethylene glycol is a synthetic, uncharged, nonbranched, hydrophilic polymer made by joining units of ethylene glycol by an ether linkage. Different molecular weight PEGs are presented and when molecular weights are <1000, PEG is liquid in nature. At higher molecular weights PEGs are solids. Polyethylene glycol is characterized as highly soluble in both water and organic solvents. The potential use of PEG as a fluid marker was identified in 1947 by Shaffer and Critchfield (1947) where they reported that PEG 4000 and 6000 were not absorbed by the rat intestine. Later, a group of scientists used PEG 4000 as a liquid marker to trace the flow of water and solutes from the reticulorumen into the omasum of sheep (Sperber et al., 1953). According to them PEG possess all the required characteristics such as chemical purity, non-absorbability, minimal toxicity, resistance to digestive enzymes, minimal effect on bacterial enzymes, and precisely measurable to be a reliable marker (Faichney, 1993). However, PEG has several limitations, such as the capacity of PEG to complex with tannin in feed and the need to measure it by a turbidimetric assay, which is affected by technique (Downes and McDonald, 1964). Despite these drawbacks, PEG is the longest used liquid marker and typically of a molecular weight of 4,000 or greater is used.

The use of liquid markers to estimate liquid passage, rumen volume, and ruminal substrate disappearance in lactating dairy cattle has been reported in several studies (Weisbjerg et al., 1998; Shingfield et al., 2008). Several studies have also used PEG as an external fecal marker to estimate total fecal output (Corbett et al., 1958; Hopson and McCroskey, 1972). An early study by Hyden (1961) has demonstrated that in sheep and goats, fluid volume as calculated from the concentration of PEG represented 95% of the total rumen water. Further studies by Ulyatt, Blaxter, and McDonald (Ulyatt et al., 1967) also reported that the mean fluid volumes determined by PEG agreed with direct measurements at slaughter in three sheep. In most of these studies animals were fed with either hay or concentrate or the mixture of both indicating that PEG is a reliable marker across different feed types.

META-ANALYSIS IN RUMINANT NUTRITION

Animal nutrition research has markedly evolved in the recent past and consequently a drastic increase in the number of publications, increasing data availability on related quantitative measurements. The range of basal plane of nutrition, various controlled and non-controlled factors among experiments and relatively smaller effects on the systems calls for the need to summarize the results from past research. Results from single experiments cannot be applied to draw conclusions or make inferences for general applicability since the particular experiments are controlled for conditions which are specific for that single experiment and the research question. The objective of these experiments is to address a specific question and hypothesis testing to a narrow subject. Often these experiments are repeated with changes to the conditions of the previous experiment but primarily focusing on a similar question. This objective of testing on generalizability and repeatability produces a hundred experiments over time attempting to answer questions in a specific area or fill in knowledge gap in that particular area. Integration of results from experiments focused on a similar question facilitates the more efficient and accurate inference making regarding the research question. Meta-analysis is one of the approaches that can be used for this integration process.

Meta-analysis can be explained as a statistical tool to summarize and combine quantitative results from independent researches on a certain topic (Glass et al., 1981) or an objective (Sauvant et al., 2008). The history of meta-analysis is dated back to the 1904 in an attempt to summarize results from independent vaccine studies regarding typhoid (Littell et al., 2008). Nonetheless, it was in the late 1970s' when social and behavioral scientists started using meta-analysis and currently in the field of animal science, we can see an exponential growth of number of published meta-analysis. In animal science meta-analysis has been successfully used to modify and create new empirical models to improve both understanding and prediction aspects and to update feed unit systems. Objectives of a meta-analysis can be 1) For global hypothesis testing, 2) For empirical modelling of biological responses, 3) For collective summarizations of measurements and, 4) In mechanistic modelling (for the estimation of parameters and initial conditions of state variables).

Limitations of Traditional Meta-analysis Approaches

According to the Web of Science, the number of publications that have applied meta-analysis in animal science shows an increase of 15% per year and is likely to continue for several more years (Sauvant et al., 2020). Although meta-analysis provides a great tool to statistically summarize previous studies several weaknesses of the approach exist. Addressing heterogeneity across experiments, defining inclusion and exclusion criteria for the selection of experiments, handling missing values are only a few major challenges that need to be addressed.

Recursive Feature Elimination

With the advancement of the technology, quantity and availability of data continue to increase but the analysis methods to handle this huge amount of data are still in the rising stage (Darst et al., 2018). Rumen is considered as a complex ecosystem with a large number of variables and therefore the data produced in rumen fermentation experiments are generally high-dimensional data. Random forest is a machine learning approach generally used to analyze high dimensional data (Darst et al., 2018) thus making it a suitable candidate to analyze rumen fermentation data. An advantage of this approach is its capacity to identify strong predictors of a specified outcome without making assumptions about an underlying model (Breiman, 2001). However, random forest does not have the ability to discover significant predictors by reducing the estimated importance scores of correlated variables in high dimensional data in the presence of correlated predictors (Gregorutti et al., 2017). To overcome this limitation Random-Forest-Recursive Feature Elimination (RF-RFE) algorithm is suggested (Gregorutti et al., 2017). The method's original goal was to make it possible for support vector machines to execute feature selection by iteratively training a model, rating features, and then eliminating the features with the lowest rankings (Guyon et al., 2002). Recursive feature elimination has become an essential tool to select the best subset of features and is gaining popularity in across various disciplines including biology, medicine, finance, manufacturing and production. In this approach it chooses the least important features for elimination (Jiang et al., 2004) and select the optimal feature subset based on the learned model and classification accuracy.

Network Analysis

In complex eco-systems, such as rumen with large number of inter-related physical, chemical, biological parameters understanding systems level phenomena is a challenge. Most of the traditional statistical analysis approaches target individual variables in complex systems, which is often insufficient to understand these eco-systems in a holistic manner (Barabási, 2012). Therefore, there is a need to analyze and make inferences about such eco-system's complex inter-relationships and network analysis has the capacity to capture these relationships and represent the eco-system holistically. In network analysis system components/ variables are identified as network nodes and relationships among nodes are named as edges. Network structure is constructed based on important information concerning the joint probability distribution of a set of variables (Cox and Wermuth, 2014). In graphical network models unconnected nodes indicate the conditional independence from all or a subset of nodes in the network and vice-versa with connected nodes (Cox and Wermuth, 2014). Network analysis approach has been widely used by biologists, mathematicians, social scientists, and computer scientists but less utilization in animal nutrition discipline so far. Few examples for network analysis usage as a tool are species in a food web (Krause et al., 2003), nodes on a computer network (Pastor-Satorras and Vespignani, 2001), proteins in metabolic pathways (Guimera and Nunes Amaral, 2005), social and behavioral sciences (Cramer et al., 2010; Robinaugh et al., 2020) and epidemiology studies (Danon et al., 2011; Gardy et al., 2011). Networks can be either directed or undirected graphical structures.

Frequentist Network Analysis

The least absolute shrinkage and selection operator (LASSO) is mostly used to estimate frequentist networks and with this operator all parameter estimates shrink toward zero (Haslbeck and Waldorp, 2015; Epskamp et al., 2018b). Therefore, LASSO has the capacity to estimate parameters and also selects which edges are included in a network. Resulting network is named as a Gaussian graphical model (GGM) which is an undirected graph (network) and equivalent to partial correlation networks (Costantini et al., 2015; Epskamp et al., 2018a) where the nodes represent the variables and the edges represent conditional dependence, or conditional correlation (Maathuis et al., 2018). In a network structure color and weight of an edge indicates its magnitude and the direction where red edges indicate negative partial correlation while blue/ green indicates positive correlations and wider and more saturated lines indicating stronger partial correlations (Epskamp et al., 2012). The method LASSO uses a tuning parameter called lambda (λ) to regulate the sparsity of the resulting network and by setting the tuning parameter to zero it simply calculates the maximum likelihood estimates of the partial correlations (Costantini et al., 2019). Selection of a value for the tuning parameter is based on the selected information criterion such as the Extended Bayesian information criterion (EBIC) (Foygel and Drton, 2010) or according to cross-validation (Krämer et al., 2009).

Accuracy and Stability of Frequentist Network

After estimating the network structure post-hoc analysis is performed to test the accuracy and the stability of the network parameters and descriptive statistics such as centrality indices based on the estimated network structure (Epskamp et al., 2017). Several methods/ steps have been applied to test the accuracy and stability of networks and those are, (A) estimation of the accuracy of edge-weights, by drawing bootstrapped CIs; (B) investigating the stability of (the order of) centrality indices after observing only portions of the data; and (C) performing bootstrapped difference tests between edge-weights and centrality indices to test whether these differ significantly from each other (Epskamp et al., 2018a).

Edge-weight accuracy is assessed by estimating the variability of edge-weights which is an estimate of 95% confidence intervals (CI) of cases. Confidence intervals are typically constructed using sampling distribution of the statistic of interest. But since obtaining such sampling distributions are difficult in complicated statistics such as centrality measures a bootstrapping method can be deployed to construct CIs (Efron, 1992). Either parametric or non-parametric bootstrapping (Bollen and Stine, 1992) can be used and it involves repeatedly estimating a model under sampled or simulated data and estimating the statistic of interest.

Centrality stability provides insights to the accuracy of the centrality indices based on subsets of the data. This measurement shows if, after re-estimating the network with fewer cases or nodes, the order of centrality indices remains the same. A case dropping subset bootstrapping method is used to estimate the stability of centrality indices. Bootstrapping can be applied to various proportions (e.g 10 %, 20% ,) of the data set and estimate correlation between the original centrality indices and those obtained from subsets. For an example, if the correlations are completely changed after dropping, say 10 % of the cases, then interpretations of centralities have less accuracy.

To determine whether a certain edge, A-B is significantly larger than another edge, A-C or whether node A's centrality is significantly greater than node B's, it is important to test for significant differences. Again, bootstrapped values of two edge-weights can be used to test if those are significantly different from each other. This can be achieved by calculating the difference between bootstrap values of one edge-weight or centrality and another edge-weight or centrality and constructing a bootstrapped CI around those difference scores. By examining whether zero is present in the bootstrapped CI, a null hypothesis may be tested in this method to determine whether the edge-weights or centralities differ from one another (Chernick, 2011).

Bayesian Network Analysis

Bayesian networks (BN), also called belief networks, Bayesian belief networks, Bayes nets, and sometimes also causal probabilistic networks, are becoming popular for modelling uncertain and complex ecosystems. The approach is immensely useful for data mining, determining and explicitly displaying the relationship among variables, representing expert knowledge and combining expert knowledge and empirical data, and identifying key uncertainties (Cheon et al., 2009; Hanea et al., 2010; Landuyt et al., 2013). Bayesian statistical approaches falls under the umbrella of artificial intelligence as a sub section of machine learning approaches and are mathematical models based on Bayes theorem and presented graphically. Each variable in this graphical structure is represented as a node, and the directed relationships between them create arcs. Probability is used by Bayesian networks to assess uncertainty since beliefs about the values of variables are stated as probability distributions, and the bigger the probability distribution, the greater the uncertainty. Several advantages of Bayesian modelling approaches are the capacity to handle missing data, potential to combine data with expert knowledge, facilitate causal relationships, provide method to avoid over-fitting of data (Heckerman, 1995), and good prediction accuracy even with small sample sizes (Kontkanen et al., 1997a). Bayesian networks have been applied in wide variety of disciplines including environmental modelling and management (Krüger and Lakes, 2015) including forecasting impacts of environmental disturbances such as fire (Dlamini, 2010) and climate change (Sperotto et al., 2017), and medical diagnoses (Arora et al., 2019). Parameter estimation in BN is performed by estimating conditional probability distribution (CPD) at each node and it depends only on its parents. This conditional probability is frequently represented by a table for discrete random variables, and using these local conditional probability tables, it is possible to compute the joint distribution of a set of variables in a unique way (Ben-Gal, 2008).

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Chapter 3 : Alternative Analytics Strategies to Complement Meta-Regression Analyses in Animal Nutrition: An Example Exploring Milk Yield and Composition

This chapter will be submitted as Sujani S., B.R. dos Reis and R.R White. Alternative Analytics Strategies to Complement Meta-Regression Analyses in Animal Nutrition: An Example Exploring Milk Yield and Composition. Journal of Animal Science, 2023.

ABSTRACT

This manuscript evaluated 2 alternative analytical approaches namely, recursive feature elimination (RFE) and additive Bayesian networking (ABN) in parallel to the traditional mixed-model-meta-analysis. Recursive feature elimination is an approach (based on random forest machine-learning method) to select features with high relative importance and fits a model while dropping features with low relative importance. Additive Bayesian networking is a method to determine an optimal directed acyclic graph and a multivariate approach using machine-learning and well adapted to study messy, highly correlated data. To evaluate how these alternative data analytics approaches might complement traditional meta-analyses, our objective was to explore the strengths and limitations of linear-mixed effect regression, RFE, and ABN in identifying relationships among diet, rumen, animal, and milk performance variables. One hundred and five (194) studies representing 705 individual treatments were used to construct the database leveraged in this study. Most relationships identified among the analysis methods were consistent with existing knowledge on dietary and ruminal variables contributing to milk production and composition. The ABN and the mixed-modelling approach aligned well in most analyses and highlighted the need for considering interactions in animal nutrition modelling. Although the ABN was a more intuitive way of depicting the complex associations among variables, the mixed-model was better suited to specific quantitative inquiry. The RFE frequently failed to identify the same important variables as the ABN and the mixed-model approach, likely due to the lack of interactions considered within that framework. Based on this analysis, future meta-analyses may benefit from inclusion of ABN as a component of exploratory data analysis or as a complementary analysis to traditional mixed models.

Keywords: *Additive Bayesian network, dry matter intake, meta-analysis, minor volatile fatty acid, recursive feature elimination, rumen pH*

INTRODUCTION

Meta-regression analysis using linear, mixed-effects models has expanded in use within the animal nutrition context over the past decade (White et al., 2016, Ungerfeld 2018, Boerman et al., 2015). This approach is now the standard for developing critical tools such as the Nutrient Requirements of Dairy and Beef Cattle (National Academies of Sciences, 2021). Despite the advantages of the meta-regression approach, there are a number of limitations which have not been thoroughly discussed within the literature. These include, among others such as slope-by-study interactions widely discussed in St-Pierre (2001), challenges in handling multicollinearity and lack of objectivity around model selection.

Linear mixed-effects models, like most multivariable regression techniques, assume independence among explanatory variables. In animal nutrition contexts, explanatory variables often include dietary percentage values, which, by definition, are correlated by calculation. The use of meta-analytical data allows for dilution of this correlation within the meta-dataset, where a plethora of different dietary interventions may reduce global correlations among variables. However, dietary variables will still be highly correlated within a study making robust cross-study associations challenging to identify and providing a potential mechanism for the slope-by-study interaction challenge highlighted by St. Pierre (2001). For example, the impact of "higher protein" in a study evaluating substitution of protein for starch may differ considerably from the impact of the same intervention in a study evaluating substitution for fiber. As a result of these tradeoffs, identification of a global response to shifting levels in one explanatory variable, without considering interactions with all other dietary variables is highly problematic and likely contributes to the often-poor model performance after omitting study effects (White et al., 2016; Gleason and

White, 2018; Souza and White, 2021). There are numerous quantitative techniques which have been explicitly designed to better account for or handle collinearity among explanatory variables. These approaches generally either seek to find parsimonious or unique solutions which avoid collinearity (Darst et al., 2018; Huang et al., 2018), or attempt to embrace the collinearity as an expected feature of the data (Alvarez et al., 2014; Finn et al., 2019). An example of the former is random forest recursive feature elimination (RFE), which iterates through deriving a random forest regression, calculating the feature importance statistics, dropping variables with the lowest importance index, and re-deriving the regression. In large data analytics problems, RFE approach has been well-recognized as a robust strategy for parsimonious model selection to minimize collinearly issues (Darst et al., 2018). Few examples of RFE utilization are to evaluate simulated pharmaco-epigenetic effect of a fictitious drug on triglyceride response (Darst et al., 2018), gene selection (Huang et al., 2018) etc. As an example of the alternative approach, additive Bayesian Networking (ABN) is directly analogous to a generalized linear model; however, it allows for all variables to be partially dependent. An ABN is estimated in two stages, the first involves learning the structure of the network from the data, while the second formalizes a quantitative description of the conditional probabilities associated with relationships included in the network structure. As such, the derivation of conditional probability distributions under the ABN paradigm allows for the collinearly among variables within the dataset to be directly considered during the model fitting process. In practice, the testing of such collinearity is a part of the data-driven structure selection process, just as the quantitative description of that collinearity is inherent in the parameter estimation step of the ABN derivation. Studies involved in antibody testing (Pittavino et al., 2017), anti-microbial resistance (Ludwig et al., 2013; Lopes et al., 2019) and veterinary epidemiology (Pittavino and Furrer, 2018; Yamaguchi et al., 2021) have successfully adopted ABN approach.

Interestingly, both RFE and ABN also provide alternative means of variable selection. A number of variable selection strategies have been employed in recent meta-analyses in the animal nutrition space, including backward elimination (Martineau et al., 2017; Gleason and White, 2018), all-possible-models (Daley et al., 2022), and forward selection (Brisson et al., 2022), among others. In each of these methods, the modeler is responsible for identifying sets of potential explanatory variables. When considering only main effects, this task is fairly easy to complete and justify; however, given the need to more comprehensively model interactions within animal nutrition, complications arise. There are few guidelines, outside the research question to be investigated, as to how many interactions should be included in animal nutrition models or the appropriate level of interaction (2-way, 3-way) that should be tested. Further, the inclusion of interactions in some variable selection approaches (i.e., all-possible-models) can result in problematic decision points or intermediate models where the interaction is considered without inclusion of the corresponding main effects. Incorporation of a more holistic, and data-driven strategy for variable selection, capable of representing interaction-like effects, would expand confidence in existing meta-analyses to support the decisions made by the modeler in retaining or omitting relationships, either in initial variable selection or during stepwise variable selection procedures. In both the RFE and the ABN frameworks, the data are used holistically to drive suggestions about important variables to include in an analysis and key relationships to consider. Complementing existing meta-analysis approaches with these data-driven structure learning strategies may help identify more robust and researcher-independent relationships. Comparison of relationships identified among methods may also provide more confidence in the strength, direction, and conditionality of associations identified.

We hypothesized that RFE and ABN have the capacity to minimize or overcome limitations in mixed-models using 4 -step backward elimination in explaining relationships among highly correlated variables. Thus, the objective of this work was to explore the strengths and limitations of mixed-models using 4 -step backward elimination, RFE, and ABN in identifying relationships among diet, rumen, and milk performance variables. The dataset used was well suited to this analysis because many associations among rumen variables (i.e., acetate and milk fat) or dietary variables (i.e., nutrient density and milk yield) are well-known at this point, meaning there is some degree of confidence in biological understanding to drive comparison among these analytical methods and discussion of their complementarity.

MATERIALS AND METHODOLOGY

Data Collection and Preparation: Literature Search Strategy and Inclusion Criteria

Search for the publications were conducted from April 2020 to April 2021 using 03 search engines, Google Scholar, Web of Science and Scopus and the publications from the year 2000 to 2020 were considered. Key words used for the search included dairy cattle, rumen, digestibility, volatile fatty acid, fermentation and milk production individually and in different combinations. Summary of the search and selection process used to identify relevant publications to be included in the meta-analyses are presented in Figure 1.

Studies were eligible to include in the database if they were 1) published in the English language in a peer-reviewed journal; 2) *in vivo* experiments which investigated at least two dietary treatments on dairy cattle; 3) reporting data on at least total and individual VFA (at least major VFA), milk yield (MY), and composition; and 4) reported least squares means and standard errors of the means (SEM) or standard deviation (SD) for response variables of interest. Data on component yields were extracted from publications when reported. When data were not reported, component yield was calculated by multiplying milk yield by milk component percentage. The proximate composition of diets used in the studies was recorded. The database leveraged 194 publications and consisted of 705 treatments. A complete list of publications used in this analysis is included in Supplementary Table 1. In addition, data on rumen pH, DMI (kg/d), and rumen ammonia nitrogen (NH_3-N; mg/L) were collected.

When data were reported in non-traditional units, conversion to a common unit was performed. For example, NH_3-N reported in mg/dL was converted to mg/L to achieve commonality among all studies reporting NH_3-N. Because the models derived include weighting by the inverse of the standard error, experimental design information was also collected from each study. Experimental designs were categorized as randomized complete block designs (RCBD) and derivatives (RCBD, RCBD with repeated measures, and RCBD with factorial arrangement of treatments), completely randomized designs (CRD), Latin square design (LSD; and all the different factorial assignments of LSD), crossover designs, and all other experimental designs to unfactorial designs. These categories of experimental design were used to contextualize reported standard errors across experimental designs, as has been done in our previous works (e.g., Souza and White, 2021).

Correlation Analysis and Model Derivation

All statistical analyses were performed using R version 4.1.2 (R Core Team, 2021). Pearson correlation coefficients between response variables and dietary, rumen fermentation and animal parameters were estimated for reference of base associations inherent within the data. The R package lme4 version 3.4.3 was used for model derivation (Bates et al., 2015). Response variables analyzed in this study were MY (kg/d), milk fat percentage (%), milk fat yield (kg/d), milk protein percentage (%), and milk protein yield (kg/d). Six sets of models with different explanatory variables were used to explain the variation in each response variable. Dietary variables, rumen fermentation variables, animal variables, solely and the combination of all variables with, and without interactions of rumen pH comprised the explanatory variables. Dietary variables included were dry matter intake (DMI, kg/d), dry matter percentage (DM %), organic matter percentage (OM %), crude protein (CP %), neutral detergent fiber (NDF %), acid detergent fiber (ADF %), starch, and fat percentage. Body weight (BW) at the beginning of the experiment and days in milking (DIM) were considered as animal variables. Concentration of total VFA (mM), molar proportions of acetate (Ac %), propionate (Pr %), butyrate (Bu %), valerate (Val %), isobutyrate (Ibu %), isovalerate (Iva %), rumen pH, and NH_3-N (mg/l) were rumen fermentation parameters. More explicit dietary variables (i.e., digestible energy, digestible nutrients, etc.) were not included due to lack of coherence in calculating these values among various decision support systems. The goal of the exploration with linear, mixed-effect models was to evaluate how rumen fermentation parameters may expand on information which could be gleaned from models leveraging only basic (measurable) data on dietary composition.

All linear, mixed-effect models were derived using a weighted linear mixed effect regression with a random study effect using a 4-step backward elimination approach as described by (Roman-Garcia et al., 2016) and (White et al., 2016) with some modifications. Since there were publications generated from the same laboratory, we assigned a unique laboratory identification number for individual laboratory and included in the model as a random variable in which the study effect was nested. In brief, during the first step of model derivation all explanatory variables in the set used in that particular model (i.e., dietary variables, rumen variables, or both, with and without interactions with ruminal pH) were included. Variables were iteratively removed by order of least significance ($P > 0.05$). When all remaining variables were significant ($P \leq 0.05$), a trend towards significance ($0.05 < P \leq 0.10$), or were a part of a significant interaction, variables that had previously been eliminated were reintroduced in reverse order of removal, one at a time, and evaluated for significant effects. In the third step, variance inflation factors (VIF) of variables included in each model were used to evaluate multicollinearity (Akinwande et al., 2015). Main effects were required to have VIF < 10 to remain in the model; however, variables included in interactions were correlated by calculation and allowed to have VFA less than 500. Main effects included in final models were checked for slope-by-study interactions. Regardless of using dietary or rumen fermentation variables, DMI was considered for all the models to help calibrate the magnitude of the response variable. Irrespective of its significance, the DMI variable was retained in all final models for all response variables. The following model was fitted for all variables.

$Yijk = \mu + \alpha i + \beta j + eijk,$

where $Yijk$ is the response variable, μ is the overall mean, αi is the vector of slopes (α) and fixed effects (i), βj is the vector of slopes (β) of random effects (j, where publication identification number was nested within the lab identification number), and $eijk$ is the residual error.

Weights used in regressions were calculated as 1 divided by the standardized, truncated standard error of the mean (SEM). Standard errors were standardized based on the mean and standard deviation within each experimental design category. In cases where errors had a substantially skewed distribution, as determined by visual assessment, these standardized SEM were truncated to remove no more than 10 % of measurements to prevent overweighting of these small-SEM observations. For observations where the SEM was missing, the observation was assigned a SEM equal to the maximum SEM reported within the dataset. This was done to ensure data were included, even if they could not be heavily weighted within the regression due to the lack of reported SEM.

Model Evaluation

The purpose of model evaluation within the present work was to explore the degree of variability in each response variable that could be explained by combinations of independent variables, for benchmarking of general model quality. Where identical sets of data were used for model derivation, models could be compared amongst one another based on their fit statistics; however, this was uncommon. The fit statistics evaluated included the standard error variance for study (σˆs), standard residual error variance (σˆe) and the ratio between σˆs / σˆe were used to assess the variability associated with individual studies relative to residual error. Values with a high ratio indicate substantial variation associated with study, which may indicate poor capacity to explain trends among studies in a prediction context. Lin's Concordance Correlation Coefficient (CCC) with the calibration for study omitted was used to evaluate model accuracy (Lawrence and Lin, 1989) and Akaike Information Criterion (AIC) was also used to assess model fit given the data.

Recursive Feature Elimination

As a complement to the traditional meta-analysis approach, we conducted a RFE to explore a parsimonious ranking of the relative importance of various explanatory variables for each response. When a model contains too many predictors, it may become more complex or develop other issues like multicollinearity and overfitting. Our objective in conducting this complementary analysis was to develop precise yet simplified sets of variables important for explaining variation in our responses. Random Forest is a machine learning approach, and it ranks the importance of each explanatory variable included in a model by constructing a collection of decision trees

(Gregorutti et al., 2017). Algorithm constructs each decision tree independently with random samples drawn from the training data using a bootstrap aggregating method. Generally, a random bootstrap sample consists of 2/3 of the total data set and use to predict test data. Recursive feature selection was selected as an alternative analysis approach to complement linear, mixed-effect modelling because it has been justified as a robust strategy to address model selection and handle correlation among variables (Gregorutti et al., 2017). Although this approach does not have the capacity to explore the nested data structure associated with a study effect and is best suited to exploring main effects in analysis, it has some notable strengths.

To perform the RFE we used the faux (DeBruine, 2021) package for simulating new variables and the caret (Kuhn, 2008) and randomForest (Liaw and Wiener 2002) packages for implementing the RFE. Random forest RFE is performed by iterating through deriving a random forest regression, calculating the feature importance statistics, dropping the variable with the lowest importance index, and re-deriving the model. First, we specified the cross validation technique as well as the random forest algorithm which then passed into the rfe function in feature selection. We chose repeated cross validation method and the RFE was constrained to allow up to all explanatory variables to be included in the final set of ideal features, and to select this set of ideal features only after identifying potential sets and testing them in a 10-fold cross-validation, repeated 5 times (Kuhn et al., 2020). We expected that the RFE would have improved robustness against multicollinearity and greater capacity to identify a robust and parsimonious set of relationships of interest within the dataset compared with the other analysis methods.

Additive Bayesian Network

An alternative approach to exploring associations within the dataset was ABN. Although traditional methods like linear mixed-effect models are highly sensitive to variance inflation or miss-parameterization due to lack of independence among explanatory variables, multivariate analytics methods are more robust against these challenges. However, linear, mixed-effect regression models have the strength that they can accurately represent the nested error structure of meta-analytical data, whereas most multivariate methods are not designed to handle these nested structures. Additive Bayesian networks provide a compromise whereby a holistic exploration of associations among variables can be explored while concurrently considering hierarchical data structures. The ABN approach is directly analogous to a generalized linear model; however, it allows for all variables to be potentially dependent. This means that the challenge associated with multicollinearity imposed on linear mixed effect models is embraced within the ABN analysis as a central assumption about data structure. Unlike the traditional use of linear mixed effects models in meta-analysis, the goal of ABN is structure discovery, namely, to identify a graphical model best describing the interrelationships driving processes within the dataset. Although these graphical models can be constructed using prior knowledge (Hay et al., 2014) for this analysis we constructed an ABN which was largely data-driven (Kratzer et al., 2020), concurrently providing an alternative means for variable section than backward elimination used in traditional meta-analysis and the RFE.

For our analysis, we used the *abn* package (Kratzer et al., 2020) to estimate the network structure. Estimation of an ABN first estimates the structure of the relationships within the data and subsequently estimates the parameters of the joint probability distribution. This joint probability distribution is represented as a directed acyclic graph (DAG), which is the visual representation of the probabilistic model. The structural priors imposed within the structure learning phase assumed that for any given daughter node, all combinations of candidate parent nodes are equally likely (i.e., we used uninformative priors). The distribution of each parameter was assumed to be gaussian. To take advantage of the value of incorporating prior knowledge on allowable structural associations, arcs to dietary composition variables from animal performance or ruminal variables were blacklisted within the structure learning phase given that we know the diet composition values were specified by experimenters, not in response to animal performance measurements. All other arcs were allowable within the network. To keep the computation tractable, the networks evaluated in building the score cache were capped at 6 parents per node. The most probable structure was selected using an exact order-based approach following Koivisto and Sood (2004). The model parameters were determined through an integrated nested Laplace approximation (Lindgren and Rue, 2015). Both the directionality and presence of associations among variables identified through the ABN and the mixed-effect regression models were compared to explore practical strengths and weaknesses of these approaches in the meta-analysis context.

RESULTS AND DISCUSSION

The objective of this work was not to develop prediction models for practical use but to evaluate alternative analytical tools alongside a traditional meta-analysis approach with a limited set of independent variables.

Description of Data

Descriptive statistics for the data are presented in Table 3-1. The diets included in this study consisted of a large variety of feeds ranging from low quality roughage to high quality total mixed rations. The database consisted of 194 published articles and each experiment consisted of at least 2 treatments making a total of 705 treatments. Out of 194 studies, 189 studies used Holstein cows, 1 study used a mix of Holstein and Brown Swiss cows and 4 studies were conducted with Jersey cows. Although 705 treatments were available for use, individual models typically relied on fewer treatments due to missing values. This was notably the case for the dietary variables, due to limited reporting of the proximate composition of the diets.

Milk Yield

The final linear, mixed-effect models for MY are reported in Table 3-3. Variability in MY was adequately explained by the models, with minimal differences among models (CCC 0.80 to 0.85). The models tended to result in substantial error variance between-study (6.02 to 6.23, Table 3-3), and minimal residual error variance (0.66 to 0.69, Table 3-3), suggesting that notable between-study variation in milk yield was left unexplained by the associations explored with dietary, rumen, or combinations of these variables. The high degree of between-study variance (Table 3-3) suggests these models would have limited applicability in naive prediction settings. Further models derived using dietary variables (Table 3-3, Model 1) and the interaction between dietary variables and rumen pH (Table 3-3, Model 4) resulted same fit statistics suggesting the interaction of rumen pH with dietary parameters were not crucial in explaining MY variations. The variables identified through RFE are depicted in Figure 3-2, and the ABN in Figure 3-7. Correlation analysis (0.766;

Table 3-2), meta-analysis (Table 3-3), RFE (Figure 3-2) and the ABN were all able to highlight the substantial importance of DMI in explaining MY responses. In the RFE which included DMI, DMI was nearly 2 times as important as any other variable in explaining MY responses (Figure 3-2, Panel B). In the mixed-model analysis, DMI was highly significant in every model. The importance of DMI on milk yield was further confirmed by the ABN, where DMI was an immediate parent of, and directly associated with MY. The fact that all analysis methods were able to identify a significant effect of DMI on MY was not unexpected because DMI and MY are widely understood to be interrelated (NRC, 2001). In previous works, both Hristov et al., (2000) and Martin and Sauvant (2002) reported moderate, positive associations between DMI and MY. Overall, the analysis methods were consistent in their capacity to highlight a critical association known to influence milk yield. This suggests that irrespective of data challenges such as multicollinearity or model selection, each analysis method should have capacity to highlight particularly strong associations such as that between DMI and MY.

Two of the 6 models identified significant, positive and negative relationships between dietary NDF and ADF percent and MY, respectively, (Table 3-3), and the RFE highlighted both variables as the 5th and 6th most important variable in explaining variability in MY. The ABN placed NDF and ADF as dietary drivers with indirect influences on other dietary variables (starch and OM), as well as the molar proportion of Pr, rumen pH and NH_3-N, which ultimately impacted MY. Through these pathways dietary NDF and ADF percent influenced milk yield, this association worked through the influence of 3 or more other nodes. The relationships between NDF and MY have been studied in numerous studies (Hammond et al., 2016; Krämer-Schmid et al., 2016; Cao et al., 2021). Even though ruminants require dietary NDF to maintain rumen functions, association between NDF and MY should be interpreted cautiously since the effect of NDF depends on the form and the degradability of NDF (Huhtanen et al., 2006). Higher percentages of dietary NDF can cause physical fill of the rumen and suppress the DMI, resulting in lowered milk production (Krämer-Schmid et al., 2016). However, when dietary NDF is higher in degradability it positively influences the daily DMI and subsequently increases milk production. According to Oba and Allen (1999) a 0.01 increase in the in vitro or in situ NDF degradability of a wide variety of forages improved daily DMI by 0.17 kg.

Given the well-established inverse relationship between dietary ADF of a diet, the negative association of ADF with MY was expected. However, the cascades in the ABN more explicitly highlight the influence of NDF on other dietary and ruminal parameters, suggesting altered fermentation conditions as a mechanism of influence. This more mechanistic interpretation of NDF as a driver of milk yield within the ABN highlights both a strength and a weakness of the approach. Although the ABN was able to highlight interesting nuances of rumen fermentation, it did not highlight a direct association among these variables. This limitation could potentially be addressed through inclusion of calculated dietary energy estimates, or through more explicit measurements of post-absorptive energy substrate availability. Interestingly, ABN was also not capable of capturing the direct positive association between Pr and MY which is well-established in theory.

Although the analysis methods tended to identify similar relationships as important in driving milk yield, there were a few inconsistencies among methods. For example, the RFE highlighted DM percent (Figure 3-2, Panel A and C) and CP (Figure 3-2, Panel B) as primary variables of interest; however, the variables were not significant in mixed models and were high-level, flow-through, intermediate variables in the ABN. The inclusion of DM percent as an important variable is somewhat non-traditional; however, it could reflect variability in the physical form of the diet. Hay-based diets would be expected to yield lower MY than silage-based total mixed rations, which are often more digestible and higher in energy. The inclusion of dietary DM percent may highlight a limitation of the RFE approach or might suggest the RFE is more sensitive to categorical shifts in associations, such as might be reflected in DM percent as an indicatory of dietary silage inclusion rates.

Dietary CP was not significant in any of the mixed-models and this finding is in agreement with previous studies showing no improvement of MY when dietary CP was increased from 16.1–16.7% to 18.4–18.9% (Broderick, 2003; Leonardi et al., 2003). The mean dietary CP value for our database was 17% which is in the range where increasing CP can have diminishing returns on production due to excess CP producing excess RDP for microbial use and ultimately elevated N excretion in urine. If more defined forms of protein, such as metabolizable protein or amino acids had been used in the analyses, it is likely these may appear as significant variables in mixed models. The higher level of CP in the ABN suggested an indirect impact on MY through other variables, which aligns with the latter concept of usage of more defined protein terms.

Dietary starch percent is also a critical indicator of energy availability in the modern lactating dairy cow diet (Koenig et al., 2003); however, it was not significant in mixed models and was the 4th variable of importance in the RFE (Figure 3-2, Panel A). Despite this, the starch dynamics in the ABN matched well with biological expectations. Starch was directly associated with ADF and CP contents of rations, suggesting some consistent tradeoffs among those nutrients due to ration formulation and feed selection. Dietary starch exhibited a positive impact on Pr through OM, resulting in a negative association between Pr and rumen pH. The inverse association revealed through this pH-mediated pathway subsequently influenced MY through NH_3-N. The negative association between rumen NH_3-N and MY was highlighted in both mixed-models and ABN, and has previously been reported in a review (Huhtanen et al., 2003) and a meta-analysis

(García, 2017). Although the mechanisms are not well understood, studies injecting ammonium salt in goats (Conrad et al., 1977) or dosing urea in the rumen of Holstein steers (Bartley et al., 1981) reported increased cellular acidification and high ammonia concentration in the carotid artery affecting the central nervous system and reduced feed intake. Hence, it is speculated that depression in MY is an indirect impact of the negative impact of DMI when ruminal NH_3-N is high (Weiss, 2015; García, 2017). In another theory, low efficiency of nitrogen utilization in ruminants due to deamination results in a high rate of ammonia production in the rumen (Chen and Russell, 1988; Flachowsky and Lebzien, 2006). When the production of ammonia exceeds the capacity of rumen microorganisms to utilize ammonia it can be detrimental for nutrient degradation and utilization that will ultimately impact MY negatively.

The RFE, ABN, and linear, mixed-effect models also did not always agree on rumen fermentation variables of importance in explaining variation in MY. The linear, mixed-effect models leveraged Pr heavily (Table 3-3), while the RFE highlighted most individual VFA with similar, and moderate levels of importance (Figure 3-2, Panel C). In the ABN none of the VFA had a direct association with MY. The ABN generated two distinct cascades, one where pH and NH_3-N were driven by the cascading effect of molar proportions of Ibu, Val, Ac and Pr, and another cascade where those same variables were driven independently by Bu molar proportions. Milk yield was conditionally dependent on NH_3-N and DMI. Although indirect and less critical than some other variables, the RFE and the ABN highlighted the capacity of minor VFA to influence MY. Valerate, Iva and Ibu have previously been shown to stimulate the growth of cellulolytic and certain non-cellulolytic ruminal bacteria (Slyter and Weaver, 1969), therefore it was expected that elevated molar proportions of these minor VFA would support elevated milk production. Further, isoacids are considered as markers of rapid fermentation of starch and sugars, suggesting increased fermentation of carbohydrates caused increased MY. Previous literature on Ibu and Iva typically identify MY is stimulated by these minor VFA; however, some work suggests a quadratic effect or non-constant responses (Felix et al., 1980; Klusmeyer et al., 1987; Liu et al., 2009). The capacity of the RFE and the ABN to identify associations of minor VFA with MY provides a framework to better explore more complex associations between fermentation conditions and the influence of minor VFA on MY. Providing this context for interpretation highlights a strength of the ABN framework in visualizing complex associations resulting in apparent inconsistencies within previous literature, and in supporting the identification of areas of future work.

Milk Fat Content and Yield

Linear, mixed models derived to explain variations of milk fat percentage and milk fat yield are reported in Tables 4 and 5, respectively. These models had moderate CCC (0.64 to 0.74; Table 3-4. Variations in milk fat percentage (%) as explained by diet, rumen fermentation, combination of both variables with and without interactions of rumen pH), with generally high ratios of error variance associated with study versus residual. Much like the conclusion drawn from the milk yield models, the mixed models resulted in fairly limited capacity to explain variability in the observed milk fat changes reported among studies, irrespective of the set of independent variables used. Although more complex models with rumen effects and interaction terms tended to result in elevated fit statistics, those same models also were derived from smaller datasets, meaning the improved fit is likely due to reduced variability in the data.

The analytical approaches differed in their capacity to highlight important relationships driven by dietary factors. Both the mixed models (Table 3-4 and Table 3-5) and the RFE (Figure 3-4 and Figure 3-4) highlighted DMI as an important variable associated with both milk fat percent and yield; however, the ABN showed an indirect association between milk fat and DMI, driven by the DMI effect on total milk yield. This indirect association is also supported by the lack of significance and negative association of the DMI term in most mixed models for milk fat percent (Table 3-4. Variations in milk fat percentage (%) as explained by diet, rumen fermentation, combination of both variables with and without interactions of rumen pH). The association between DMI and milk fat yield is likely driven by elevated DMI supporting greater availability of metabolizable energy, and elevated total milk yields. The capacity of the ABN and mixed models to differentiate between the DMI effects on milk fat yield versus percent highlight capacity to interpret findings from these methods in a more mechanistic fashion.

Three of the 6 mixed models identified dietary starch and 1 model identified fat as significantly associated with milk fat percent (Table 3-4. Variations in milk fat percentage (%) as explained by diet, rumen fermentation, combination of both variables with and without interactions of rumen pH) but both variables had negative associations. The RFE also highlighted the importance of starch and fat percentages, with dietary fat content among the higher-ranked dietary characteristics (Figure 3-3). Other than DMI, the ABN treated all other dietary parameters as intermediate variables, driving milk fat percent through a cascade eventually resulting in rumen pH, NH_3-N, and MY. Dietary fat in the ABN drove an independent cascade of VFA molar proportions, which eventually also resulted in modulating rumen pH-mediated effects on milk production and subsequently milk composition. The network clearly separates individual physiological responses driven by protein, carbohydrate, and fat, which match well with biological expectations. Dietary fat can affect milk fat percentage, though in a manner dependent on inclusion level and source (DePeters and Cant, 1992). Indeed, a meta-analysis on the association identified notable variation of milk fat responses to different sources of dietary fat (Rabiee et al., 2012).

Although we did not explicitly evaluate different fat sources, the ABN results suggest that some of this variation identified in previous meta-analyses may be due to interactions with ruminal factors driven by dietary carbohydrate and protein balances. Further, the direction of associations identified within the ABN show pathways for both direct and inverse associations of dietary fat and milk fat percent. The ABN and the mixed models also identified plausible relationships linking dietary starch to milk fat. In the mixed models, an inverse association was identified, and within the ABN, starch was indirectly associated with Pr, which was inversely associated with rumen pH and, subsequently, milk fat percent. Starch also was associated with depressed rumen pH, facilitating increased Pr through both substrate and pH-mediated relationships.

The ABN and the mixed-models (Table 3-4) identified the primary ruminal variables influencing milk fat percentage directly included Ac, Bu, and indirectly rumen pH and NH_3-N. Most absorbed Bu is converted to beta-hydroxybutyrate in the rumen wall, which is also a critical substrate for milk fat synthesis (Tyasi et al., 2015). The RFE identified Bu as the 6th variable of importance. In ABN DIM had a negative impact on both Ac and Bu and consequently impacted milk fat. Though the direct association between the stage of lactation and rumen VFA is not extensively researched a possible relationship can be presumed linking DIM, Ac, Bu with milk fat. The mean DIM in our database is 109 which represents the latter part of early lactation and the onset of mid lactation. During this period, the cow starts to shift from an extreme negative energy balance to a positive energy balance thus decreasing lipolysis and uptake of fatty acids mobilized from body fat and this leads to decreased fat synthesis in udder (Buttchereit et al., 2010). Since Ac and Bu have a positive association with milk fat percentage and the reduction of milk fat with increasing DIM, the negative association of DIM with Ac and Bu matches biological expectation. Although the RFE (Figure 3-3) identified acetate as the most important factor, Pr was 4th overall and rumen pH did appear as the most important variable when combination of variables were considered (Figure 3-3, Panel C). It is likely that the inability to consider conditional probabilities or interactions limited the capacity of the RFE to highlight associations within the complex rumen environment. Both the mixed-models and the ABN were able to capture the influence of the Ac:Pr ratio on milk fat percentage through considering inverse associations between Pr and milk fat and direct associations between Ac and milk fat. Although both approaches were also able to capture interactions with rumen pH, the ABN is somewhat more intuitive in displaying those interactions. Irrespective of this preference of authors toward the graphical conveyance of information, both analytics approaches were able to highlight acetate as a major driver of milk fat percent and yield. This was expected because Ac provides the substrate for oxidation and precursors for de novo synthesis of milk fat. Further, Ac stimulates lipogenesis in the bovine mammary gland through transcriptional regulation (Jacobs et al., 2013).

Milk Protein Content and Yield

The mixed-models derived to explain the variation of milk protein percent and yield generated moderate (0.60 to 0.70) and higher (0.80 to 0.85) CCC values after removing study effects (Table 3-6 and Table 3-7). Milk protein yield models performed better in terms of CCC and exhibited less variation between studies relative to residual variation. Much like the milk fat models, those which were derived from smaller datasets had greater fit statistics, suggesting that reducing variability in the dataset was likely a primary driver of differences observed among model fit statistics.

Three of the mixed-models and RFE for milk protein percent again highlighted DMI percent as major dietary driver. The RFE suggested fat percent as the second leading driver of milk protein percent (Figure 3-5, Panel A). The ABN suggested milk protein percent was driven through the cascade influencing milk yield and inversely associated with milk yield. It is well documented that DMI, and milk protein yield are interrelated (NRC, 2001). Increased DMI can improve milk protein by 0.2 to 0.3 percentage units, as a result of increased energy intake. As such, the inverse association observed in the ABN lacks clear justification, though may reflect diluting synthesized milk protein quantities as greater volumes of milk were produced.

The mixed models highlighted Pr and Ibu as important ruminal factors influencing milk protein (Table 3-6 and Table 3-7). Interestingly RFE identified NH_3-N as an important variable by selecting as the 3rd and 2nd important factors (Figure 3-5, Panel B and C) and ABN showcased a negative indirect association between NH_3-N and milk fat in which MY was an intermediate. Negative impact (Huhtanen et al., 2003) and no impact (García, 2017) of NH_3-N from grass and alfalfa silage, respectively were reported previously. In the same study Huhtanen et al., (2003) observed that for milk protein yield a quadratic effect was a better fit than a linear model. Nonetheless, the negative association between NH_3-N and milk protein most likely resulted due to the reduced MY. In the ABN, except of Iva all other VFA were part of cascades influencing milk protein percent. The mixed-models and the ABN showed capacity for Ibu to have direct or indirect associations with milk protein percent, which is supported by previous literature where supplementation of Ibu showed a quadratic effect on milk protein percent and milk protein yield (Liu et al., 2009). Most literature on branched chain VFA support positive associations with milk protein percent (Wang et al., 2019); however, the rumen pH-mediated effects shown in the ABN and mixed-models provide a potential explanation for those studies which do not show this direct association. Again, the strength of the mixed-models and ABN in explaining complex interrelationships among variables is highlighted through analysis of ruminal factors associated with milk protein percent.

CONCLUSIONS

Existing animal nutrition models, along with decades of nutrition experiments, provide a strong body of reference material to explore how different, complementary analytical methods highlight associations among diet, rumen fermentation indicators, and dairy cow performance. Although the use of mixed-model meta-analysis has gained tremendous popularity over the past decade as a tool to explore complex relationships within meta-datasets, the approach suffers from challenges such as variable selection bias and poor capacity to handle multicollinearity. Additive Bayesian networking and RFE were investigated as alternative strategies to extract critical relationships from meta-datasets which may be more robust to the challenges associated with variable selection and multicollinearity. Throughout analysis of the dataset, a consistent strength of the mixed-model approach and the ABN approach was their capacity to explore interactions within meta-datasets. Because the RFE focused on main effects only, it often identified different sets of important variables, failing to appropriately capture the complexity of relationships within the dataset. However, a big strength of RFE approach is the ability to highlight when a set of variables have roughly the same importance. This indicates the potential use of other dimensionality reduction techniques (like PCA) to simplify variables with a similar importance to a single variable prior to deriving ABN or mixed-models. Further work is needed to confirm whether such a conclusion from the RFE analyses is warranted. Further, although variable selection bias was a concern within the traditional meta-analysis approach, many of the important associations and interactions identified within the ABN were also supported by the mixed-models. This suggests that, provided careful specification and appropriate variable selection procedures, the mixed model technique described herein can be quite robust in extracting important features from meta-datasets. That said, as meta-analysis is used more and more frequently to explore associations which are not clearly understood from existing models and literature, there are likely many examples of application contexts where it may be challenging to select the best set of main effects and interactions to test. In these contexts, supporting traditional meta-analyses with ABN may help justify starting variable selection choices and support more data-driven analyses. As a final trade-off apparent from the analysis, the ABN provides a simplified but extremely intuitive means of exploring holistic associations within a complex, intercorrelated data environment. Gleaning equivalent understanding from a mixed-model or set of mixed-models (as presented here), often requires considerable simulation exercises focused on reductionist questions which may oversimplify biology or extend the data beyond the ranges provided in derivation. As such, there is notable value in the ABN approach in communicating associations identified within meta-analyses. That said, the mixed-models provide a tool which is easier to use quantitatively, should direct quantitative estimation of a response be the goal of the meta-analysis exercise. Based on the complementary expertise of these approaches, and the similarity in results obtained form this analysis, ABN and linear mixed-effects models should be combined in future meta-analysis exercises to better explore structural associations within the data both visually and quantitatively.

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FIGURES

Figure 3-1. Prisma flow diagram of the study selection process

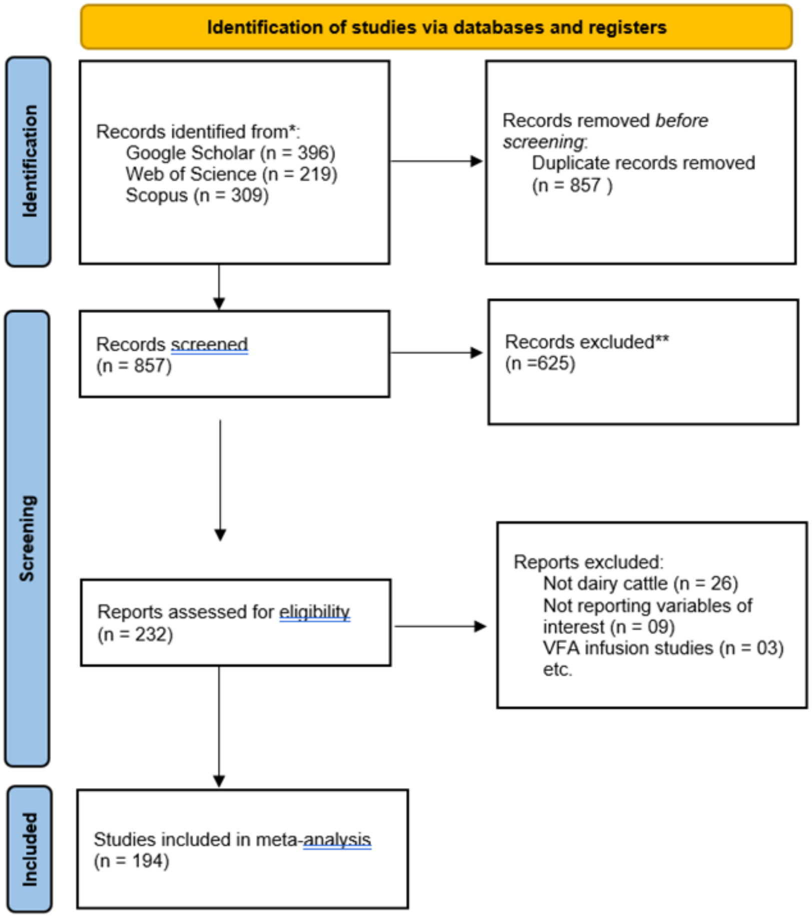
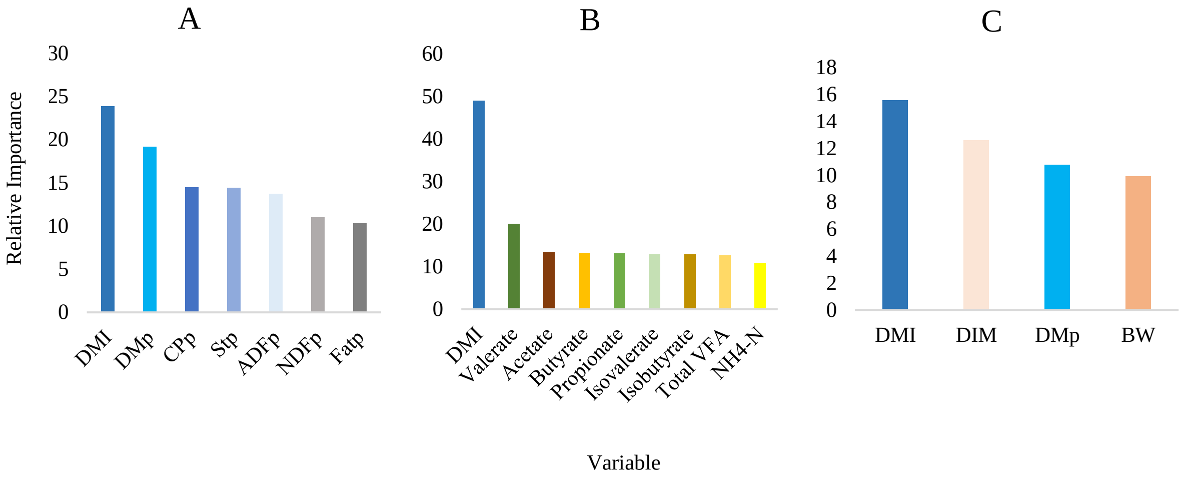
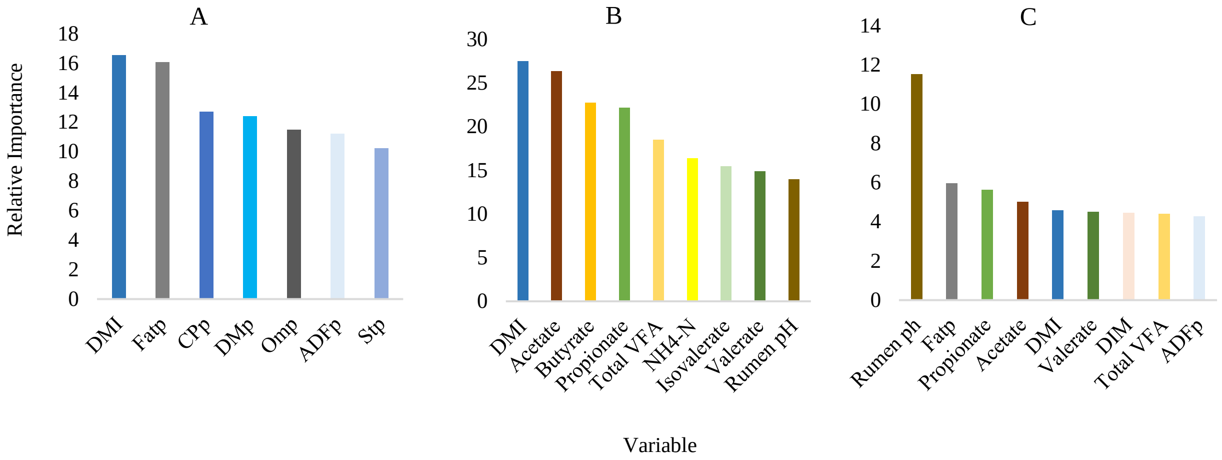


Figure 3-2. Variation in milk yield (kg/d) explained by dietary parameters (A), rumen fermentation parameters (B), and the combination of dietary, rumen fermentation and animal parameters (C). The y-axis reflects the relative importance (unitless) of individual variable



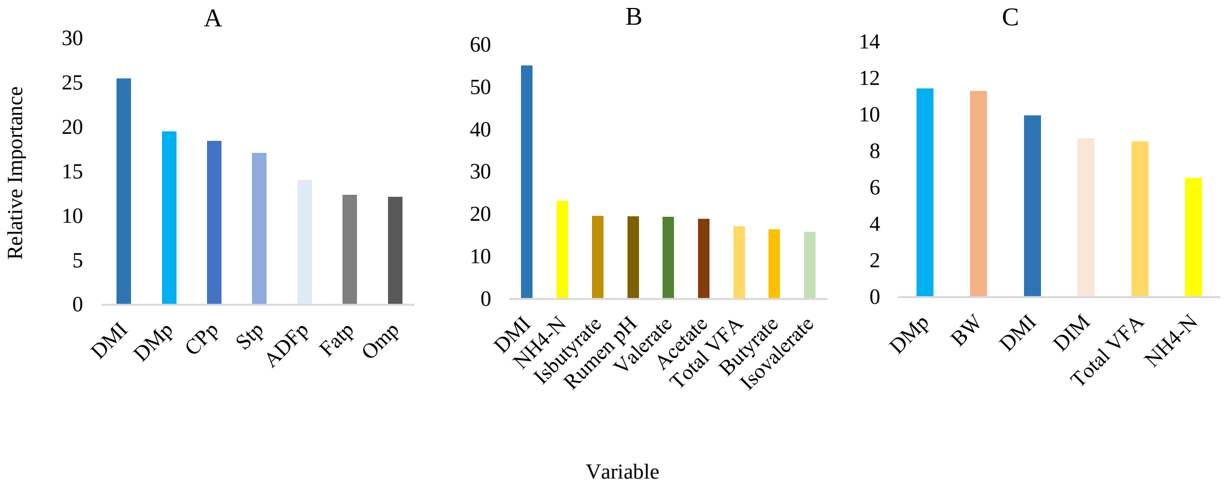
Variables from highest to least importance on milk yield as estimated through random-forest RFE analysis are illustrated. DMI: dry matter intake, DMp: dry matter percentage, Fatp: fat percentage, ADFp: acid detergent fiber percentage, NDFp: neutral detergent fiber percentage, CPp: crude protein percentage, Stp: starch percentage, DIM: days in milk, BW: body weight

Figure 3-3. Variation in milk fat content (%) explained by dietary parameters (A), rumen fermentation parameters (B), and the combination of dietary, rumen fermentation and animal parameters (C). The y-axis reflects the relative importance (unitless) of individual variable



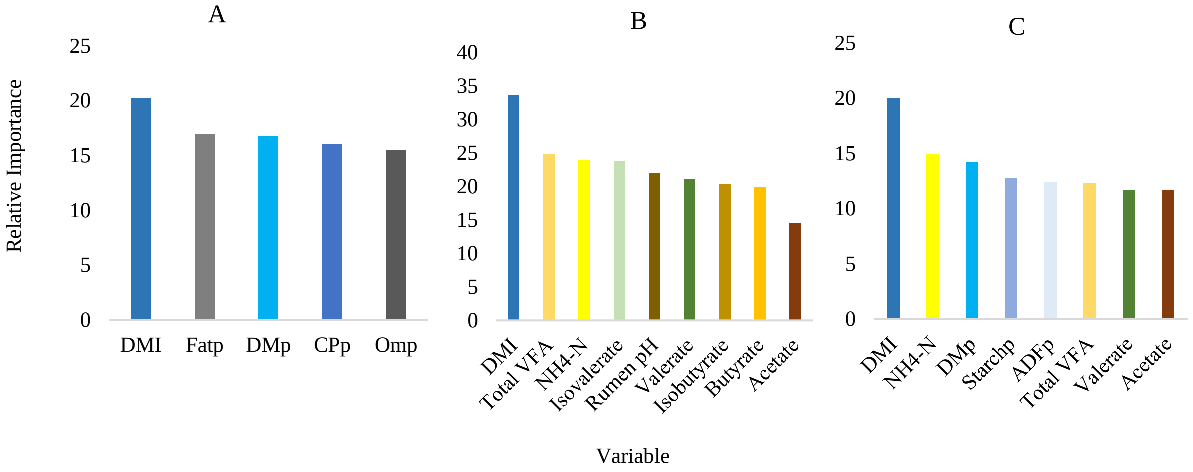
Variables from highest to least importance on milk yield as estimated through random-forest RFE analysis are illustrated. DMI: dry matter intake, Stp: starch percentage, DMp: dry matter percentage, Fatp: fat percentage, ADFp: acid detergent fiber percentage, OMp: organic matter percentage, CPp: crude protein percentage, DIM: days in milking

Figure 3-4. Variation in milk fat yield (kg/d) explained by dietary parameters (A), rumen fermentation parameters (B), and the combination of dietary, rumen fermentation and animal parameters (C). The y-axis reflects the relative importance (unitless) of individual variable



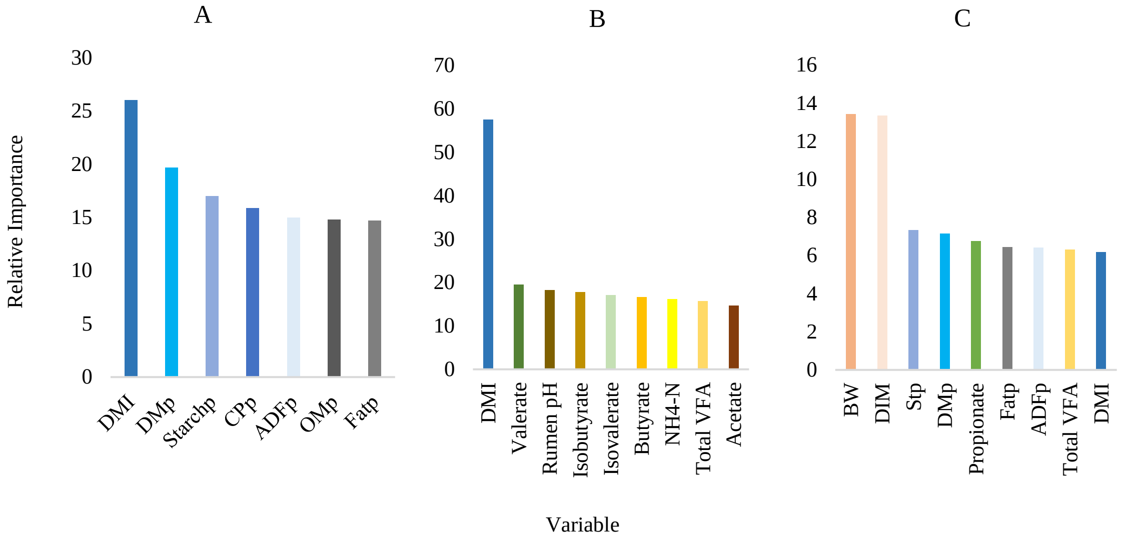
Variables from highest to least importance on milk yield as estimated through random-forest RFE analysis are illustrated. DMI: dry matter intake, DMp: dry matter percentage, Fatp: fat percentage, ADFp: acid detergent fiber percentage, OMp: organic matter percentage, CPp: crude protein percentage, DIM: days in milking, BW: body weight

Figure 3-5. Variation in milk protein content (%) explained by dietary parameters (A), rumen fermentation parameters (B), and the combination of dietary, rumen fermentation and animal parameters (C). The y-axis reflects the relative importance (unitless) of individual variable



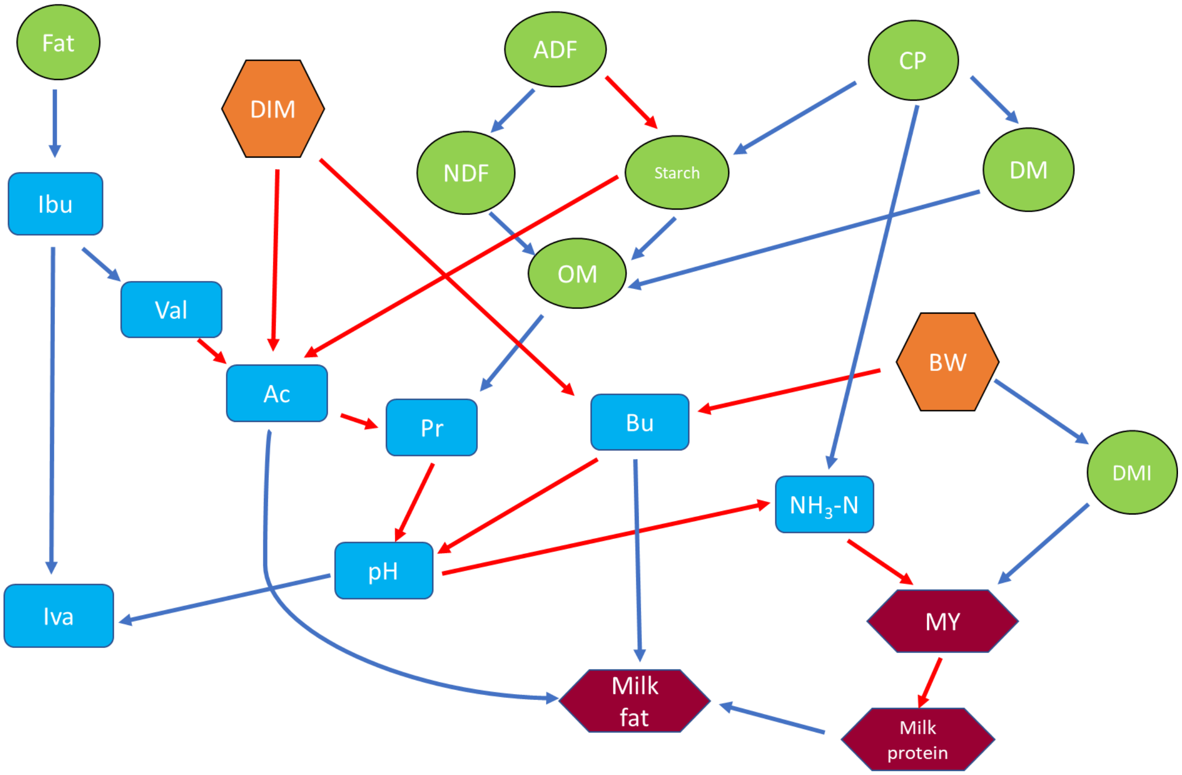
Variables from highest to least importance on milk yield as estimated through random-forest RFE analysis are illustrated. DMI: dry matter intake, DMp: dry matter percentage, Fatp: fat percentage, ADFp: acid detergent fiber percentage, OMp: organic matter percentage, CPp: crude protein percentage

Figure 3-6. Variation in milk protein yield (kg/d) explained by dietary parameters (A), rumen fermentation parameters (B), and the combination of dietary, rumen fermentation and animal parameters (C). The y-axis reflects the relative importance (unitless)



Variables from highest to least importance on milk yield as estimated through random-forest RFE analysis are illustrated. DMI: dry matter intake, DMp: dry matter percentage, Fatp: fat percentage, ADFp: acid detergent fiber percentage, OMp: organic matter percentage, CPp: crude protein percentage, DIM: days in milking, BW: body weight

Figure 3-7. Additive Bayesian network of dietary and rumen parameters to explain milk yield and composition.



The parameters are represented by nodes and the edges represent the relationships among parameters. The directionality of the probabilistic relationship between the two parameters is indicated by the direction of the edge. For example, milk fat percentage was conditionally dependent on acetate molar proportions and milk protein percentage. The directionality of the association among variables is shown by the color of the edge (red edges reflect indirect associations while black edges reflect direct associations).

TABLES

Table 3-1. Summary of data yielded by literature search that was utilized for analyses.

Variables	**Variable**	**n**	**Mean**	**SD**	**Minimum**	**Maximum**
Animal	Starting BW, kg	594	624	72.3	380	768.5
	DIM, d	649	109	50.22	15	328
	DMI, kg/d	705	22.2	3.92	9.40	31.8
	MY, kg/d	705	32.5	7.71	10.1	52.5
	Milk fat, %	705	3.83	0.287	2.29	4.91
	Milk fat yield, kg/d	705	1.16	0.263	0.358	1.95
	Milk protein, %	705	3.16	0.242	2.55	3.96
	Milk protein yield, kg/d	705	1.14	0.220	0.302	1.70
Diet characeteristics[1]	DM, %	431	56.1	11.6	30.6	95.6
	OM, %	474	91.1	3.38	53.9	97.7
	CP, %	612	17.0	1.85	7.31	24.6
	NDF, %	605	32.8	5.46	11.9	63.6
	ADF, %	468	20.4	3.87	5.5	36.5
	Starch, %	332	23.4	6.94	0.50	46.2
	Fat, %	419	3.93	1.42	1.50	11.6
Rumen fermentation	Total VFA, mM	705	114	21.1	29.4	174
	Ac, %	705	61.9	5.30	40.8	84.5
	Pr, %	705	22.2	3.14	13.9	33.4
	Bu, %	705	12.1	2.25	5.80	21.9
	Val, %	492	1.82	0.62	0.55	4.50
	Iva, %	416	1.50	0.68	0.11	4.80
	Ibu, %	405	1.01	0.57	0.04	5.40
	Rumen pH	689	6.20	0.35	2.36	7.6
	NH_3-N, mg/L	624	129.9	53.2	19	366

[1] All expressed as a percentage on a DM basis except DM, which is expressed on an as-fed basis

Table 3-2. Correlation of dietary, animal and rumen fermentation variables with response variables

Variable	**Milk yield, kg/d**	**Milk Fat content, %**	**Milk fat yield, kg/d**	**Milk protein content, %**	**Milk protein yield, kg/d**
DMI, kg/d	0.750 (<0.001)	-0.380 (<0.001)	0.625 (<0.001)	-0.320 (<0.001)	0.750 (<0.001)
DM, %	-0.100 (0.050)	-0.169 (0.001)	-0.178 (<0.001)	-0.110 (0.027)	-0.110 (0.037)
OM, %	0.012 (0.816)	-0.002 (0.957)	-0.058 (0.267)	0.125 (0.016)	0.019 (0.710)
CP, %	0.128 (0.015)	-0.167 (0.001)	0.039 (0.449)	0.080 (0.125)	0.159 (0.002)
NDF, %	-0.276 (<0.001)	0.207 (<0.001)	-0.183 (<0.001)	0.079 (0.132)	-0.290 (<0.001)
ADF, %	-0.311 (<0.001)	0.287 (<0.001)	-0.152 (0.003)	0.098 (0.066)	-0.317 (<0.001)
Starch, %	0.200 (<0.001)	-0.427 (<0.001)	-0.066 (0.215)	-0.166 (0.001)	0.170 (0.001)
Fat, %	0.118 (0.023)	-0.245 (<0.001)	-0.044 (0.401)	-0.196 (<0.001)	0.063 (0.227)
Total VFA, mM	0.066 (0.205)	-0.044 (0.395)	0.077 (0.142)	0.079 (0.131)	0.130 (0.012)
Ac, %	-0.145 (0.005)	0.372 (<0.001)	0.100 (0.056)	<0.001 (0.989)	-0.138 (0.008)
Pr, %	0.227 (<0.001)	-0.387 (<0.001)	-0.030 (0.567)	-0.105 (0.044)	0.210 (<0.001)
Bu, %	-0.069 (0.191)	0.224 (<0.001)	0.079 (0.129)	0.154 (0.003)	-0.021 (0.686)
Val, %	0.068 (0.260)	-0.380 (<0.001)	-0.179 (0.003)	-0.132 (0.029)	0.038 (0.533)
Iva, %	-0.019 (0.854)	-0.129 (0.046)	-0.103 (0.114)	0.004 (0.942)	-0.010 (0.877)
Ibu, %	-0.108 (0.089)	-0.156 (0.013)	-0.200 (0.001)	0.033 (0.597)	-0.102 (0.108)
Rumen pH	-0.170 (0.001)	0.190 (<0.001)	-0.073 (0.167)	-0.012 (0.816)	-0.190 (<0.001)
NH_3-N, mg/L	-0.214 (<0.001)	0.075 (0.171)	-0.189 (<0.001)	0.170 (0.001)	-0.193 (0.003)
Starting BW, kg	0.446 (<0.001)	-0.212 (<0.001)	0.357 (<0.001)	-0.102 (0.074)	0.486 (<0.001)
Parity	0.181 (0.211)	0.143 (0.326)	0.327 (0.023)	-0.359 (0.011)	0.054 (0.713)
DIM, d	-0.349 (<0.001)	0.136 (0.012)	-0.330 (<0.001)	0.393 (<0.001)	-0.253 (<0.001)

Pearson correlation coefficient between variables is presented. P values are given in parenthesis.

Table 3-3. Variations in MY (kg/d) as explained by diet, rumen fermentation, combination of both variables with and without interactions of rumen pH

Item	**Diet**	**Rumen**	**Diet + Rumen**	**Diet*rumen pH**	**Rumen*rumen pH**	**Rumen*rumen pH + Diet*rumen pH**
Model	1	2	3	4	5	6
Intercept	11.0 (<0.001)	9.83 (0.010)	10.7 (0.007)	11.0 (<0.001)	8.97 (<0.001)	8.97 (<0.001)
Starting BW			0.014 (0.002)			
DMI	0.941 (<0.001)	1.00 (<0.001)	1.01 (<0.001)	0.941 (<0.001)	0.938 (<0.001)	0.938 (<0.001)
NDF	0.107 (0.013)			0.107 (0.013)		
ADF	-0.172 (0.003)			-0.172 (0.003)		
Ac		0.071 (0.025)				
Pr		0.094 (0.013)			0.0817 (0.002)	0.0817 (0.002)
Bu			-0.208 (0.001)			
Rumen pH		-1.02 (0.008)	-1.23 (0.001)			
NH_3-N		-0.006 (0.009)	-0.007 (0.004)			
Fit Statistics						
n	462	608	529	462	693	693
CCC	0.80	0.83	0.85	0.80	0.81	0.81
AICc	1989	2599	2252	1989	2994	2994
$\hat{\sigma}_s$	4.18	4.10	4.09	4.18	4.12	4.12
$\hat{\sigma}_e$	0.686	0.660	0.657	0.686	0.685	0.685
$\hat{\sigma}_s / \hat{\sigma}_e$	6.09	6.25	6.23	6.09	6.02	6.02

P values are given in parentheses.

CCC = Concordance correlation coefficient; AICc = corrected Akaike information criterion; σˆs = standard deviation for study; σˆe = standard deviation for error.

Table 3-4. Variations in milk fat percentage (%) as explained by diet, rumen fermentation, combination of both variables with and without interactions of rumen pH

Item	**Diet**	**Rumen**	**Diet + Rumen**	**Diet*rumen pH**	**Rumen* rumen pH**	**Rumen*rumen pH + Diet*rumen pH**
Model	1	2	3	4	5	6
Intercept	4.35 (<0.001)	5.50 (<0.001)	2.20 (<0.001)	-11.7 (0.001)	-13.7 (0.002)	3.37 (<0.001)
DMI	-0.014 (0.088)	-0.009 (0.187)	-0.006 (0.269)	-0.026 (0.017)	0.296 (0.003)	-0.003 (0.613)
NDF				0.480 (<0.001)		
Starch	-0.016 (<0.001)			-0.017 (<0.001)		-0.008 (0.003)
Fat				-0.069 (0.002)		
NDF*rumen pH				-0.076 (<0.001)		
DMI * rumen pH					-0.048 (0.002)	
Ac			0.025 (<0.001)		0.218 (<0.001)	0.018 (<0.001)
Pr		-0.034 (<0.001)			-0.0293 (<0.001)	-0.026 (<0.001)
Val		-0.173 (<0.001)				
Rumen pH		-0.093 (0.049)		2.67 (<0.001)	2.76 (<0.001)	
NH_3-N					-0.009 (0.041)	
NH_3-N*rumen pH					0.018 (0.036)	
Ac*rumen pH					-0.033 (<0.001)	
Fit Statistics						
n	329	473	587	235	608	327
CCC	0.71	0.70	0.66	0.72	0.64	0.74
AICc	109	125	182	87.40	175	64.0
$\hat{\sigma}_s$	0.335	0.369	0.347	0.363	0.350	0.300
$\hat{\sigma}_e$	0.105	0.102	0.103	0.0965	0.098	0.093
$\hat{\sigma}_s / \hat{\sigma}_e$	3.17	3.59	3.53	3.76	3.57	3.22

P values are given in parentheses.

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

Table 3-5. Variations in milk fat yield (kg/d) as explained by diet, rumen fermentation, combination of both variables with and without interactions of rumen pH

Item	**Diet**	**Rumen**	**Diet + Rumen**	**Diet*rumen pH**	**Rumen* rumen pH**	**Rumen*rumen pH + Diet*rumen pH**
Model	1	2	3	4	5	6
Intercept	0.527 (<0.001)	0.618 (<0.001)	-0.080 (0.392)	-3.93 (<0.001)	-8.72 (<0.001)	-0.080 (0.392)
DMI	0.032 (<0.001)	0.036 (<0.001)	0.034 (<0.001)	0.022 (<0.001)	0.04 (<0.001)	0.034 (<0.001)
DM	-0.004(<0.001)					
NDF	0.004 (0.008)			0.136 (<0.001)		
Starch				-0.003 (0.016)		
Ac		0.005 (<0.001)	0.007(<0.001)		0.14 (<0.001)	0.007 (<0.001)
Pr		-0.009 (<0.001)				
Val					0.80 (0.005)	
Rumen pH		-0.064 (<0.001)		0.738 (<0.001)	1.36 (<0.001)	
NDF*rumen pH				-0.021 (<0.001)		
NH_3-N		-<0.001 (0.031)				
Ac*rumen pH					-0.02 (<0.001)	
Val*rumen pH					-0.13 (0.005)	
NH_3-N*rumen pH					0.001 (<0.001)	
Fit Statistics						
n	421	608	693	327	420	693
CCC	0.77	0.80	0.79	0.70	0.78	0.79
AICc	-679	-1059	-1167	-544	-677	-1167
$\hat{\sigma}_s$	0.164	0.16	0.172	0.152	0.17	0.172
$\hat{\sigma}_e$	0.037	0.03	0.037	0.034	0.03	0.037
$\hat{\sigma}_s / \hat{\sigma}_e$	4.40	4.65	4.66	4.43	4.93	4.66

P values are given in parentheses.

CCC = concordance correlation coefficient; AICc = corrected Akaike information criterion; $\hat{\sigma}$s = standard deviation for study; $\hat{\sigma}$e = standard deviation for error.

Table 3-6. Variations in milk protein content (%) as explained by diet, rumen fermentation, combination of both variables with and without interactions of rumen pH

Item	**Diet**	**Rumen**	**Diet + Rumen**	**Diet*rumen pH**	**Rumen*rumen pH**	**Rumen*rumen pH + Diet*rumen pH**
Model	1	2	3	4	5	6
Intercept	3.31 (<0.001)	2.59(<0.001)	3.20 (<0.001)	7.41 (<0.001)	2.63 (<0.001)	2.81 (<0.001)
DIM			0.0017 (<0.001)			
DMI	0.002 (0.553)	0.013 (<0.001)	<0.0003 (0.947)	0.004 (0.276)	0.013 (<0.001)	0.008 (0.042)
CP				-0.244 (<0.001)		
NDF				0.005 (0.042)		
ADF	-0.007 (0.001)		-0.008 (<0.001)	-0.006 (0.006)		
Fat	-0.020 (0.006)		-0.0202 (0.006)	-0.0186 (0.005)		-0.021 (0.009)
CP*rumen pH				0.040 (<0.001)		
Pr		0.007 (0.001)			0.007 (0.001)	0.069 (0.019)
Iva		0.065(<0.001)				
Ibu					0.066 (0.006)	0.007 (0.013)
Rumen pH				-0.698 (<0.001)		
Fit Statistics						
n	323	409	303	321	409	252
CCC	0.66	0.63	0.70	0.66	0.60	0.65
AICc	-470	-565	-430	-444	-545	-288
$\hat{\sigma}_s$	0.154	0.177	0.145	0.149	0.189	0.235
$\hat{\sigma}_e$	0.041	0.039	0.041	0.040	0.040	0.042
$\hat{\sigma}_s$ / $\hat{\sigma}_e$	3.73	4.53	3.48	3.72	4.78	5.61

P values are given in parentheses.

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

Table 3-7. Variations in milk protein yield (kg/d) as explained by diet, rumen fermentation, combination of both variables with and without interactions of rumen pH

Item	**Diet**	**Rumen**	**Diet + Rumen**	**Diet*rumen pH**	**Rumen*rumen pH**	**Rumen*rumen pH + Diet*rumen pH**
Model	1	2	3	4	5	6
Intercept	0.451(<0.001)	0.082 (0.058)	0.082 (0.058)	2.078 (0.001)	1.37 (0.059)	0.082 (0.058)
DMI	0.034 (<0.001)	0.035 (<0.001)	0.035(<0.001)	0.034 (<0.001)	-0.096 (0.012)	0.035 (<0.001)
DMI*rumen pH					0.021 (<0.001)	
DM	-0.002 (0.010)			-0.002 (0.019)		
ADF	-0.005 (0.027)			-0.075 (0.019)		
ADF*rumen pH				0.013 (0.026)		
Pr		0.006 (<0.001)	0.006 (<0.001)		0.083 (0.002)	0.006 (<0.001)
Val					-0.029 (0.027)	
Iva					0.027 (0.028)	
Rumen pH				-0.259 (0.010)	-0.211 (0.075)	
Pr*rumen pH					-0.012 (0.004)	
Fit Statistics						
n	317	693	693	314	399	693
CCC	0.85	0.83	0.83	0.84	0.80	0.83
AICc	-639	-1551	-1551	-621	-790	-1551
$\hat{\sigma}_s$	0.06	0.09	0.09	0.06	0.09	0.09
$\hat{\sigma}_e$	0.03	0.029	0.029	0.03	0.031	0.03
$\hat{\sigma}_s$ / $\hat{\sigma}_e$	2.07	3.028	3.02	2.98	2.98	3.02

P values are given in parentheses.

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

Table 3-8. Publications used to extract data to prepare the database for the meta-analysis.

Number	Publication
1	Abrahamse, P. A., Vlaeminck, B., Tamminga, S., and Dijkstra, J. 2008. The effect of silage and concentrate type on intake behavior, rumen function, and milk production in dairy cows in early and late lactation. J. Dairy Sci. 91: 4778–4792. doi.org/10.3168/jds.2008-1350
2	Agle, M., A. N. Hristov, S. Zaman, C. Schneider, P. Ndegwa, and V. K. Vaddella. 2010. The effects of ruminally degraded protein on rumen fermentation and ammonia losses from manure in dairy cows. J. Dairy Sci. 93: 1625-1637. doi.org/10.3168/jds.2009-2579.
3	Aguerre, M. J., Capozzolo, M. C., Lencioni, P., Cabral, C., and Wattiaux, M. A. 2016. Effect of quebracho-chestnut tannin extracts at 2 dietary crude protein levels on performance, rumen fermentation, and nitrogen partitioning in dairy cows. J. Dairy Sci. 99: 4476–4486. doi.org/10.3168/jds.2015-10745
4	Aguerre, M. J., Wattiaux, M. A., Powell, J. M., Broderick, G. A., and Arndt, C. 2011. Effect of forage-to-concentrate ratio in dairy cow diets on emission of methane, carbon dioxide, and ammonia, lactation performance, and manure excretion. J. Dairy Sci. 94: 3081–3093. doi.org/10.3168/jds.2010-4011
5	Aikman, P. C., Henning, P. H., Humphries, D. J., and Horn, C. H. 2011. Rumen Ph and fermentation characteristics in dairy cows supplemented with Megasphaera Elsdenii NCIMB 41125 in early lactation. J. Dairy Sci. 94: 2840–2849. doi.org/10.3168/jds.2010-3783
6	Alamouti, A. Asadi, M. Alikhani, G. R. Ghorbani, and Q. Zebeli. 2009. Effects of inclusion of neutral detergent soluble fibre sources in diets varying in forage particle size on feed intake, digestive processes, and performance of mid-lactation Holstein cows. Anim. Feed Sci. Technol. 154: 9-23. doi.org/10.1016/j.anifeedsci.2009.07.002.
7	Arriola, K. G., S. C. Kim, C. R. Staples, and A. T. Adesogan. 2011. Effect of fibrolytic enzyme application to low-and high-concentrate diets on the performance of lactating dairy cattle. J. Dairy Sci. 94: 832-841. doi.org/10.3168/jds.2010-3424.
8	Avila, C. D., DePeters, E. J., Perez-Monti, H., Taylor, S. J., and Zinn, R. A. 2000. Influences of saturation ratio of supplemental dietary fat on digestion and milk yield in dairy cows. J. Dairy Sci. 83(7), 1505–1519. doi.org/10.3168/jds.s0022-0302(00)75023-5
9	Bargo, F., D. H. Rearte, F. J. Santini, and L. D. Muller. 2001. Ruminal digestion by dairy cows grazing winter oats pasture supplemented with different levels and sources of protein. J. Dairy Sci. 84: 2260-2272. doi.org/10.3168/jds.S0022-0302(01)74673-5.

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11 Bayat, A. R., Laura Ventto, Piia Kairenius, T. Stefański, Heidi Leskinen, Ilma Tapio, Enyew Negussie, Johanna Vilkki, and K. J. Shingfield. 2017. Dietary forage to concentrate ratio and sunflower oil supplement alter rumen fermentation, ruminal methane emissions, and nutrient utilization in lactating cows. Transl. Anim. Sci. 1: 277-286. doi.org/10.2527/tas2017.0032.

12 Beauchemin, K. A., S. M. McGinn, C. Benchaar, and L. Holtshausen. 2009. Crushed sunflower, flax, or canola seeds in lactating dairy cow diets: Effects on methane production, rumen fermentation, and milk production. J. Dairy Sci. 92: 2118-2127. doi.org/10.3168/jds.2008-1903.

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18 Benchaar, C., Petit, H. V., Berthiaume, R., Ouellet, D. R., Chiquette, J., and Chouinard, P. Y. 2007. Effects of essential oils on digestion, ruminal fermentation, rumen microbial populations, milk production, and milk composition in dairy cows fed alfalfa silage or corn silage. J. Dairy Sci. 90: 886–897. doi.org/10.3168/jds.s0022-0302(07)71572-2

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Chapter 4 : Network Analysis to Evaluate Complexities in Relationships Among Fermentation Variables Measured Within Continuous Culture Experiments

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ABSTRACT

The objective of this study was to leverage a frequentist (ELN) and Bayesian learning (BLN) network analyses to summarize quantitative associations among variables measured in 4 previously published dual flow continuous culture fermentation experiments. Experiments were originally designed to evaluate effects of nitrate, defaunation, yeast, and/or physiological shifts associated with pH or solids passage rates on rumen conditions. Measurements from these experiments that were used as nodes within the networks included concentrations of individual volatile fatty acids, m*M* and nitrate, NO_3^-,%; outflows of non-ammonia nitrogen (NAN, g/d), bacterial N (BN, g/d), residual N (RN, g/d), and ammonia N (NH_3-N, mg/dL); degradability of neutral detergent fiber (NDFd, %) and degradability of organic matter (OMd, %); dry matter intake (DMI, kg/d); urea in buffer (%); fluid passage rate (FF, L/d); total protozoa count (PZ, cells/ml); and methane production (CH_4, mmol/d). A frequentist network (ELN) derived using a graphical LASSO (least absolute shrinkage and selection operator) technique with tuning parameters selected by Extended Bayesian Information Criteria (EBIC) and a BLN were constructed from these data. The illustrated associations in the ELN were unidirectional yet assisted in identifying prominent relationships within the rumen that were largely consistent with current understanding of fermentation mechanisms. Another advantage of the ELN approach was that it focused on understanding the role of individual nodes within the network. Such understanding may be critical in exploring candidates for biomarkers, indicator variables, model targets, or other measurement-focused explorations. As an example, acetate was highly central in the network suggesting it may be a strong candidate as a rumen biomarker. Alternatively, the major advantage of the BLN was its unique ability to imply causal directionality in relationships. Because the BLN identified directional, cascading relationships, this analytics approach was uniquely suited to exploring the edges within the network as a strategy to direct future work researching mechanisms of fermentation. For example, in the BLN acetate responded to treatment conditions such as the source of N used and the quantity of substrate provided, while acetate drove changes in the protozoal populations, non-NH_3-N and residual N flows. In conclusion, the analyses exhibit complementary strengths in supporting inference on the connectedness and directionality of quantitative associations among fermentation variables that may be useful in driving future studies.

Key words: Bayesian learning network, EBIC-LASSO, Gaussian graphical model, rumen fermentation, volatile fatty acids.

INTRODUCTION

Attempts at modelling to describe nutrient digestion and substrate fermentation to various end products [predominantly to volatile fatty acids (VFA)] in the rumen have a decades-long history, dating back to at least 1970 (Baldwin, 1995). Since that time, scientists have leveraged different approaches for prediction, beginning with simple empirical representations and moving to more precise and knowledge-driven, dynamic, and mechanistic (Kebreab et al., 2009) modelling to predict this complex environment while accounting for numerous physical, chemical, and biochemical interactions. Most of the models related to ruminant nutrition are applied to formulate diets, to integrate knowledge of ruminant digestion and metabolism, or to predict fermentation end products like VFA (Dijkstra et al., 1992, Bannink et al., 2006) and methane (CH_4) (Gregorini et al., 2013; Huhtanen et al., 2015, Bannink et al., 2016). Empirical models are formulated using classical regression approaches that provide a powerful statistical method to estimate quantitative associations among two or more variables of interest. Despite being robust and versatile, these regression analyses suffer from a number of drawbacks, including applicability only within the derivation data space, poor capacity to handle collinearity, reliance on researcher-selected variables and relationships, and limited ability to address mechanistic quantitative associations within data. Mechanistic models, on the other hand, make a better attempt to understand underlying metabolic processes and are believed to provide a more reliable prediction outside the calibration domain. Nevertheless, these traditional modelling approaches rely on accurate expert construction to depict the quantitative associations among variables and often incompletely account for variable variance and measurement errors (Baldwin 1995; Reed et al., 2015). As a promising complement to traditional models, network analysis methods are becoming popular given their capacity for holistic exploration of all possible quantitative associations among variables, including the potential strength and directionality of those quantitative associations in complex environments. Network analysis has been successfully used in different ecosystem studies including marine environments (Lina-Mendez et al., 2015), soil ecosystems (Barberan et al., 2012), and the human gut (Baldassano and Bassett 2016); however, use of this investigatory tool to evaluate quantitative associations within the rumen environment has been limited.

As researchers continue to investigate the quantitative associations among ruminal fermentation variables, traditional statistical approaches will likely continue to fall short as a method of effectively identifying, exploring, and quantifying these associations. The growing interest in network analysis can be attributed to the tendency towards system-level or holistic approaches in place of traditional reductionism in natural science research (Miller 2000, Mazzocchi 2012, Fardet and Rock 2015) and also to the recent advancement in the availability of high-throughput data collection, storage, and analysis tools. Further to these advantages, novel multivariate modelling approaches, such as Bayesian learning network (BLN) modelling, may potentially reveal new insights compared to classical statistical approaches by explicitly examining the implied causation of complex quantitative associations in the rumen. A BLN is an illustration of joint probability over a set of variables representing probabilistic relationships among variables (Pearl, 2011; Nemzek et al., 2015).The probabilistic inference of the model can be used to infer/suggest implied causation (we preferred the term ‘implied causation’ for the present work because it provides more clarity to the network approach we conducted) or dependence of relationships instead of causality (Scutari, 2009). Nevertheless, BLNs are not necessarily causal since it is difficult to justify the causality only with the directionality and typically causality needs to be inferred with additional assumptions and analytics (Pearl, 2000; Spirtes et al., 2000). Bayesian networks are becoming popularized across many disciplines given their potential to capture linear and nonlinear associations, and stochastic relationships among many variables (Nemzek et al., 2015). In recent works, BLN has been successfully used to study gene regulatory networks in immune systems (Behdani et al., 2019), to estimate inference of gene regulatory networks from RNA-Seq time series data (Thorne, 2018), to explore disease risks in veterinary epidemiology (Kratzer et al., 2020), to manage mastitis on dairy farms (Horpiencharoen et al., 2019), and to explore community structure in ecological modelling (Arhonditsis et al., 2006; Marcot et al., 2006). Further, BLN have the unique capacity to handle missing data (Heckerman, 2008) and depict incomplete knowledge about the biological system.

Despite the aforementioned advantages of the Bayesian networking, the objectivity of the network varies with different prior specifications. Tightly specified priors will reduce the ability of the network to be informed exclusively by the data. In contrast, relying primarily on the posterior probability of associations is a solution to minimize the subjectivity and collinearity of the network since the posterior probability distribution associated with an edge is calculated only based on the available data (Arora et al., 2019). This is also the case when estimating the edge weights within a BLN and contributes to the advantage of BLN in utilizing incomplete datasets. Because of the simplified treatment of quantitative associations among nodes, if a study has observations on 2 measurements but not the third, it could be used to inform the network edge associated with those 2 measurements, whereas in deriving most traditional models, the observation would be omitted because the third node measurement was not available. In this way, the BLN approach allows for more complete and flexible use of partial observations (Scutari, 2020). Additionally, the BLN approach intuitively encapsulates both conditionally dependent and conditionally independent quantitative associations among variables and explores these possible associations through the framework expectation that all variables may be partially dependent on one another (Pensar et al., 2016). This approach relaxes the requirement of independence among explanatory variables, which is a common challenge in exploring highly correlated environments like the rumen (Ramazi et al., 2021). A final advantage of BLN is its potential to learn from data, expert knowledge, or from a combination of both (Ramazi et al., 2021). Collectively, the capacity to represent uncertainty (Havron et al., 2017), combine information from different sources, and integrate prior expert knowledge (Ramazi et al., 2021) makes BLN a promising candidate to explore relationships among rumen fermentation variables. As such, there is a need to test BLN and other network analysis strategies for their suitability in representing quantitative associations from a comprehensive but consistent dataset as an initial test case to evaluate the sensibility, coherence, and strengths/weaknesses of these analytics approaches.

We hypothesized that rumen fermentation variables are highly interrelated, and these quantitative associations can be holistically explored with network analysis. To test this hypothesis, the objective of this study was to leverage two network analyses to characterize quantitative associations among degradable nutrient supplies, nitrogen dynamics, and volatile fatty acid concentrations from 4 continuous culture (CC) fermentation experiments conducted within a single laboratory. Utilizing data generated in a single lab minimized experimental variation which is ideal for comprehension and comparison of different networking approaches, particularly because no between-study adjustments were used to control for study-to-study variation. Continuous culture data were used because the CC system offers several advantages over in vivo experiments, including ability to test a large number of treatments with sufficient replication; less demand for time; reduced cost; and ability to test higher inclusion levels of feed additives. In comparison to the batch culture system, fermentation end products in the CC system can be removed to allow maintenance of a relatively stable fermentation for prolonged periods

(Czerkawski and Breckenridge, 1977). Another advantage of using CC data for the current study is its ability to better represent the in vivo situation in comparison to batch culture (Brandao et al., 2020). Despite these challenges, the nature of CC trials varies considerably throughout the literature and most of the data generated by these trials was used to explore similar conditions and similar research questions. Although this provides the benefit of reducing the possible study-to-study variability, it also likely resulted in limited or narrowed scope for exploring mechanisms driving fermentation within these networks.

MATERIALS AND METHODS

Experimental Design and Treatments

Data were obtained from four dual flow CC rumen fermentation experiments conducted in a single laboratory at The Ohio State University. Experiments are briefly summarized as follows and key details are outlined in Table 4-1. In experiment 1 (Wenner et al., 2017), treatments evaluated control pH (ranging from 6.3 to 6.8) or low pH (ranging from 5.8 to 6.3) factorialized with low (2.5%/h) or high (5.0%/h) solids passage rate (k_P). Experiment 2 (Wagner et al., 2018) consisted of treatments factorializing either corn or wheat starch sources with either a soybean meal control or fermented whey product. In the third experiment, control or supplemental live yeast was factorialized with a negative control or nitrate at 1.5% of dietary DM fed (Welty et al., 2019). And in the fourth experiment, treatments were isonitrogenous and included substrate with either supplemental urea or nitrate (NO_3^-) factorialized with faunated versus defaunated fermenter contents (Wenner et al., 2020).

From the experimental data, 18 nodes of interest were identified and included VFA concentrations [acetate (Ac), propionate (Pr), butyrate (Bu), valerate (Val), isobutyrate (IBu) and isovalerate (IVa) which is a mixture of IVa and 2-methylbutyrate]; flow of non-ammonia nitrogen (NAN); nitrate % (NO_3^-); flow of bacterial N (BN); ammonia nitrogen (NH_3-N, g/d); flow of residual nitrogen (RN); CH_4 production; dry matter intake (DMI); degradability of neutral detergent fiber (NDFd) and organic matter (OMd); fluid flow (FF); urea %, and total protozoa (PZ) concentration. Within network analysis, a node is defined as a variable of interest, and the quantitative association between two nodes is referred to as an edge (Hevey, 2018). Prior to the construction of networks the data for all variables were normalized using the scale function in R.

Construction of the EBIC-LASSO Network

Construction of the EBIC graphical LASSO network (ELN) was performed using R Statistical Software (R Core team, 2020). The network structure was estimated as a Gaussian graphical model (GGM) (Epskamp et al., 2018), which was categorized under the pairwise Markov Random Field using the graphical least absolute shrinkage and selection operator (LASSO) in combination with extended Bayesian information criterion (EBIC) for model selection (Foygel and Drton, 2010). Estimation of the ELN was completed using the bootnet package (Epskamp et al., 2018).

Structure learning in GGM can be accomplished using different algorithms and few can be named as conditional covariance threshold test (CMIT) (Anandkumar et al., 2012), clustering by inferred models of evolution (CLIME) (Cai et al., 2016), and LASSO (Tibshirani, 1996). Of the numerous Frequentist approaches to network with derivation, we selected the ELN approach to enhance the prediction accuracy, parsimonious property (Fu and Knight, 2000), interpretability, and generalizability, and to minimize the error associated the small data set employed in the current study (Epskamp et al., 2017). The ELN approach minimizes the errors in the usual sum of squares by applying a penalty that bounds the total sum of the absolute values of the edges. Further, we chose to use the ELN approach because it has been identified as the most appropriate, interpretable, and simple networking approach for cross-sectional, continuous data (Lauritzen, 1996) due to its analysis of partial correlations (Costantini et al., 2015). Furthermore, the ELN leverages the inverse of a covariance matrix rather than modelling the covariance matrix directly, which helps to minimize errors in edge weight estimates. Although the ELN approach is most commonly applied within the literature due to these advantages, future work should consider further exploration of other structure learning approaches which have strengths in identifying chain and grid structures in relationships among nodes (Wang et al., 2020).

The ELN approach produces a collection of networks, rather than a single network, which vary in complexity from a complete network (every node is connected with all other nodes) to an empty network (no connections between nodes). A tuning variable, lambda (λ), was applied to control the degree to which regularization was applied over these networks. Lambda was selected to minimize the EBIC (Chen and Chen, 2008), which has been shown to work particularly well in identifying the true network structure (Foygel and Drton, 2010; van Borkulo et al., 2014). The choice of λ depends on the objective of the study and to the relative importance of the number of associations identified versus the strength of associations. Lower λ results in a network with more edges, including possible spurious ones, and can be useful in early exploratory and hypotheses generating research, whereas higher λ will restrict the number of edges in favor of those with the greatest confidence. We allowed the algorithm to estimate all possible networks and it generated several networks correspondent with λ value ranging from 6.63 with 0 edges to 0.07 with 136 edges (Supplementary Figure 1). The decision of an edge as a spurious or true is somewhat arbitrary and up to the researcher. Ultimately, the network associated with λ=0.59 generated by the algorithm was selected by the researcher. The selection was performed based on the biological understanding of rumen fermentation and practicability and feasibility of interpretation of associations. For reference, two additional example networks (lambda = 0.75 and 0.46, Supplementary Figures 2 and 3) were used to generate centrality plots (Supplementary Figures 4 and 5) to demonstrate this stability of network interpretation among lambda values.

Evaluation of Accuracy and the Stability of the ELN

After estimating the network structure, the accuracy and stability of the network were determined based on a number of indices. First, network accuracy was evaluated using centrality indices from the qgraph package (Epskamp et al., 2012): degree/strength centrality, betweenness centrality, and closeness centrality (Epskamp et al., 2018). Next, the stability of the strength centrality indices was determined as an indicator of network stability. Strength centrality was selected as a focus because it shows much more stability than other centrality measurements and is therefore a more reliable indicator of network stability. Stability of the strength centrality estimates was determined using a correlation stability (CS) coefficient, which is estimated through case-dropping subset bootstrapping. The stability of the strength centrality estimate is interpreted as the maximum proportion of cases that can be dropped to retain with 95 % certainty. In a situation when the model is highly stable, larger proportions of data can be dropped without needing to adjust the network structure. In cases with notable instability, very few datapoints can be omitted before the network structure is no longer supported. As a third evaluation approach, we estimated edge-weight variability by calculating confidence intervals (e.g., 95% CI) for each edge-weight estimate using a non-parametric bootstrapping approach (Efron, 1979) leveraging the bootnet package (Epskamp et al., 2018). This approach was used because bootstrapping allows for calculating the sampling distribution of the edge-weight estimates. Finally, after determining the edge-weight and node centrality confidence intervals, we performed bootstrap difference tests to assess significant differences between edge weights or node centrality measures. This estimation allowed us to explore quantitative associations among rumen variables and within the variables, which were more robustly supported by the data.

Construction of Bayesian Learning Network (BLN)

The BLN analysis was performed using the bnlearn (Scutari, 2009) package to determine the graphical structure of BLN and estimate the network parameters. The resulting networks were graphically displayed using the Rgraphviz package (Hansen et al., 2021). A directed acyclic graph (DAG) was created with a score-based learning algorithm (Hill Climbing). Within the DAG, each node corresponds to a variable in data and each edge represents the conditional dependencies among pairs of variables. The graphical structure of BLN must be a cascading structure, not a cyclic structure that contains loops, because the presence of loops violates the conditional factorization of the joint probability distribution. This non-existence of feedback loops in BLN is a critique when the model is used to illustrate biological systems, especially because biological systems function as a response to feedback loops. Though BLN has few drawbacks, advantages BLN can offer outweighs most of these critiques and few are discussed here. Bayesian networking combines both quantitative and qualitative knowledge of available data, expert/prior knowledge, and probabilistic modelling to provide a simple yet holistic interpretation of a complex system (Zhang and Arhonditsis, 2008). Variables or sets of random variables in the system are represented by the nodes in the network while directed arcs represent conditionally independent quantitative associations between nodes. Any given variable in a BLN can play dual roles as both a response and an explanatory variable within the same network. This capacity of BLN (i.e., accounting for multiple dependencies) provides flexibility in estimating network structures.

Learning of a BLN can be categorized into two main components as (1) parametric learning, in which estimation of conditional probabilities is performed, (2) structural learning in which network structure is established. Structure learning tends to be more challenging than parameter estimation (Heckerman, 2008). To address the challenge associated with learning the structure of the network as supported by the data, several structure learning algorithms have been proposed. These algorithms fall into 3 general categories: constraint-based, score-based, or a hybrid approach including both constraint- and score-based elements (Beretta et al., 2018). Conditional independence tests serve as the basics for constraint-based methods, whereas score-based methods are established following a score function and a search procedure (Acid et al., 2004). Elaborating more on these two approaches, constraint-based approaches build a graph structure based on identifying pairs of nodes not found to be independent based on statistical tests (often a log-likelihood ratio test). Score-based methods maximize the measure of fit between the data and the DAG (Beretta et al., 2018). Hence, given the differences in underlying principles among these approaches, network structures derived through different methods may differ considerably (Acid et al., 2004). Although there are some advantages to constraint-based algorithms, most of the developed structure learning algorithms are score-based. In practice, score-based algorithms tend to be more robust, with higher accuracy in data-sparse settings, which is commonly the case in animal nutrition research. In this work, the network structure was estimated using a hill climbing, score-based algorithm that attempted to minimize the Bayesian information criterion (BIC) to find the best model from a set of random variables. Being a heuristic search algorithm, hill climbing possesses the capacity to add, drop, and change the direction of arcs, thereby modifying the network until an ideal BIC is achieved. Hill climbing adds and removes edges one at a time, which assists in maintaining acyclicity of the network while attempting to maximize the network score (Shaughnessy and Livingston, 2006).

After structure learning, the next step in deriving the BLN was parameter learning. Because all variables used in this assessment represented continuous data, the parameter learning process centered on determination of regression coefficients for each variable relative to its parents. In contrast, parameter learning for categorical variables relies on developing conditional probability tables. All parameters were assumed to follow normal distributions, and parameters were estimated through a maximum likelihood approach for each node based on its parent nodes, as identified in the DAG.

Evaluation of the BLN

Stability of the BLN models was evaluated using the network score and cross validation. In the k-fold cross-validation approach used for network assessment, observations of the network are split into k-subsets, and each subset is used as an evaluation set, while the remaining data (i.e., training set) is used to estimate associations. Evaluation of the network score and cross-validation are recommended approaches for more unbiased estimates of a network’s goodness of fit. The confidence in the quantitative associations within the network was evaluated using arc strength and accuracy (Concordance Correlation Coefficient; CCC) (Lawrence and Lin, 1989) of predicting individual nodes. Arc strength measures the strength of the probabilistic relationship depicted by the arcs of a BLN and is considered a fundamental tool to evaluate confidence in the structure of a BLN. The CCC reflects the capacity of the network to explain variability in each individual node, given known information on all other nodes.

RESULTS AND DISCUSSION

Continuous-culture systems are a subcategory of rumen simulation techniques which have several advantages over in vivo experiments. Continuous culture experiments allow researchers to control conditions for treatments in ways that cannot be controlled in vivo and typically lowers residual variation. Although the CC system offers advantages over other in vitro and in vivo experiments, it does not completely mimic actual rumen fermentation conditions (Hristov et al., 2012; Brandao and Faciola, 2019). Differences in absorption, differences in fluid and particle dilution (passage) rates, and feed intake per rumen volume exist between two approaches. As such, the results presented in this work are mostly applicable to CC systems and should not necessarily be inferred to represent in vivo dynamics.

Comparison of Network Attributes

For coherence of discussion, we will first review the structure and evaluation of the two networks, along with the use of network analysis tools to identify and compare important nodes and edges. After this discussion, we will explore biological inferences that can be drawn from networking approaches.

Structure of ELN and BLN

Figure 4-1 shows the ELN structure generated as a GGM. This network was selected using a λ of 0.59. This λ was used because the goal of the work was to create a network with a minimum number of spurious edges while maximizing the number of true edges. Although the generated network contains many edges connecting different rumen fermentation variables, the implied causality of these quantitative associations is unspecified within the ELN approach. The EBIC-

LASSO approach does, however, specify information about the directionality of associations. There is some conflict within the literature about the interpretation of these directional associations as causal associations (Nadkarni and Shenoy, 2001; Heckerman, 2013); therefore, we select the term implied causality to reflect cautionary inference based on directionality of associations. Figure 4-2 depicts the network derived using the BLN approach, describing the network quantitatively as a DAG. Much like the ELN, the BLN network includes a number of edges, and the location of nodes highlights their suggested role within the network. For example, BN and NH_3-N appear to be driving the system, whereas RN seems to be a “passenger”, responding to changes in NH_3-N. In contrast, Ac concentration is a highly central variable, both responding to elements of the system and driving other elements. Although many of the quantitative associations identified among these two networking approaches are similar, the BLN approach provides a more interpretable structure because directionality of edges allows for cautious exploration of implied causality of relationships. Furthermore, the BLN also has fewer associations, allowing for more targeted assessment of those associations that are most supported by the data. The ELN, however, has the strength of showing feedback loops within the system, which are well supported by current understanding of biology. Comparing each approach in light of their strengths allows for more robust system analysis and evaluation of relationships supported in the data. Further, with both networks we observed a lack of depiction of associations between variables which are typically presented in *in vitro* or *in vivo* systems (i.e., no direct associations between major VFA or CH_4 and Ac or Pr in the ELN and no association between Ac, Pr, PZ with CH_4 in the BLN). A probable explanation for this observation would be the effect of forced parsimoniousness on network which produces more associations through intermediates rather than direct associations.

Strength, Importance, and Stability of Nodes and Edges within the Networks

Evaluation of accuracy and stability of ELN leverages centrality indices (Figure 4-3), stability of centrality (strength) indices (Figure 4-4), calculation of edge weights (Figure 4-5), and performing bootstrapped difference tests of edge-weights (Figure 4-6). Centrality indices, which are shown as standardized z-scores (Figure 4-3), include strength (i.e., the strength of the quantitative association associated with each node), betweenness (i.e., the number of quantitative associations for which nodes are intermediary), and closeness (i.e., how close nodes are to all other nodes within the network). These indices help interpret the ELN because they depict the various dominance measures for nodes within the network. For example, acetate shows a higher centrality in all the indices, indicating its significance in the network across relationship types. By comparison, NAN flow, which also had higher closeness and betweenness centralities, was involved in a number of relationships and served as an important intermediate within the network. The specific biological inferences driven by these indices are discussed in the Biological Inference section.

Figure 4-4 illustrates the stability of strength centrality in the ELN, which reflects the stability of the strength of quantitative associations explored within the network when calculated with different percentages of the base sample. Figure 4-4 shows a noticeable drop in the correlation between the subsample estimate and the estimate from the original sample as the percentage of the sample included in the estimates decreases, indicating only moderate stability. The estimated strength stability for the current network was 0.35, suggesting that 35% of the total cases can be dropped and still sustain the network at 95% certainty. In behavioral studies, the desired centrality strength coefficient typically is set at 0.7 by default (Cohen, 1977). However, for simulation studies, the centrality strength coefficient above 0.25 is commonly interpreted as acceptable and above 0.5 as preferable. Therefore, although our centrality strength coefficient in this network was lower than desired, it is still within an acceptable range as defined by previous literature (Epskamp et al., 2018).

Edge weights (Figure 4-5) with CI that do not overlap with 0 are unique or interpretable as different from other edge weights, whereas those which do not overlap with 0 are indistinguishable in terms of strength or importance from other quantitative associations identified within the network (Figure 4-1). Networks with a large number of quantitative associations of similar importance can reflect overfit or nonspecific networks. Due to the careful selection of λ, the selected ELN yielded numerous CI that did not overlap with 0. Bootstrapped difference tests (Figure 4-6) of node strength reflect the involvement of a node within the network and were calculated as the sum of the weighted number of all associations of a specific node. Figure 4-6 illustrates that the majority of nodes significantly differ from other nodes and these illustrations are in agreement with the centrality measurement (Figure 4-3) and edge weight measurements (Figure 4-5). In context, this statistic infers that the reported nodes are not redundant, implying they have unique and individual roles within the network. Selection of the BLN structure was performed with a score-based, hill-climbing learning algorithm (Cooper and Herskovits, 1992). Although minimum description length (MDL) (Lam and Bacchus, 1994), BIC, Akaike's information criterion (AIC), and Bayesian Dirichlet equivalent score (BDeu) (Suzuki, 2017) have been used as scoring functions, we used the BIC for network evaluation. The estimated network score for the BLN was -1021.00 (unitless). Suzki (2017) suggested that a network score of this range represents moderate capacity to explain variability within the data. This moderate ability is not unexpected for a variety of reasons. First, the continuous culture data used for network establishment in this study do not represent the full scope of physiological relationships within microbial fermentations. Further, fermentation measurements often result in substantial variation (e.g., have limited precision); therefore, the data have errors that will influence the capacity of the network to explain variability inherent in the data. This known variability is also part of the justification for selecting data based on continuous culture studies as a preliminary exploration of network approaches because sources of variability such as passage rates and flows are measured manually rather than using markers in CC studies compared with in vivo studies, helping to partially improve precision of estimates. The expected loss in this network after performing k-fold cross-validation was 34.6 (unitless). The expected loss is interpreted as the change in expected log likelihood as the source data change among folds. Though a standardized value for expected loss is not established, generally the lower the expected loss value, the better, because this indicates greater stability. The expected loss in the current network (34.6) relative to the log likelihood of the network (-1021), suggests the network was fairly robust against changes in source data.

Quantitative associations in a BLN are evaluated using different metrics than those used in the ELN. The primary strategy for evaluating the stability and strength of quantitative associations identified within BLN is to evaluate the arc strength (Table 4-2). Arc strength values provide both the strength and the directionality of the relationship. More than 250 possible arc strength values were generated but only selected higher (strength above 0.90), average (0.7 to 0.9), and low (below 0.2) arc strength values are reported in Table 4-2 for practicality. The majority of the quantitative associations with low arc strengths were automatically excluded during the structure-learning phase of the network development. The final network consisted of arcs with higher strength values, indicating that the network was built upon the strongest quantitative associations within the data.

Predictability of individual nodes is another method of evaluating the accuracy of a BLN and associated CCC values for each node are reported in Table 4-2. The majority of the nodes exhibited CCC greater than 0.90, indicating the majority of variability in these nodes was explained by the network. Exceptions included FF, NH_3-N, CH_4, and PZ, which had lower CCC values, suggesting lower capacity to explain variability in these measures. Each of these variables has important biological or experimental elements, which may explain the lower CCC. Fluid flow has more influence on concentration of VFA and less involvement in causal relationships. The low CCC of NH_3 can be explained because the concentration of NH_3-N is the net of production and assimilation and was not actually varied intentionally by treatment. Methane and Pz probably have very high variability between fermenters and studies due to differences in inoculum and how they were controlled in the individual studies. The generally high predictive capacity within the BLN, as well as clear explanation for why some variables would have poor capacity, supports the idea that this networking strategy may be robust in exploring data structures in complex environments like the rumen.

Biological Inference from Networks

Due to the extent of quantitative associations identified within the networks, this discussion will focus on those which were strong, stable, and repeatable among network types. These quantitative associations supported by the network are broadly categorized into three groups: 1) associations among VFA; 2) associations among different forms of N; and 3) associations among VFA or nitrogen sources and other variables such as DMI, OM and NDF.

Associations Among Volatile Fatty Acids

Acetate was a central node in both networks and yielded significant quantitative associations with nodes representing N sources and nutrient degradability. Acetate is the predominant VFA in typical rumen fermentation patterns (Esdale et al., 1968, Sutton et al., 2003,) (except for rare occasions of higher Pr in high concentrate beef diets); hence, its dominant role and associations with other variables were expected. The ELN network suggested strong inverse associations between Ac and urea, NO_3^-, and NAN. Strong positive associations between Ac and RN, IBu, and IVa were also apparent within the ELN network. The BLN highlighted Ac as a highly central node, which responded to shifts in Val, DMI, urea, and NO_3^-, and drove changes in PZ, NAN, RN, and IBu. Importantly, Ac was inversely associated with N variables in both networks. The inverse relationship between Ac and dietary NO_3^- is sensible because this non-protein N source cannot be completely or efficiently leveraged for microbial growth, which is expected to drive VFA production. The inverse relationship between NO_3- and Ac can be further explained by the H_2 usage of NO_3- for reduction hence decreasing Ac synthesis. Urea is known to be readily available for microbial use and the inverse relationship we observed here was unexpected. Indeed, in a previous study using dual-flow continuous culture systems, rapidly available N limited microbial growth, leading to lower Ac production (Devant et al., 2001). Within the ELN, a weaker, but direct association between BN and Ac was identified, supporting this known influence of microbial growth on Ac production. Within the BLN, the same association was more mechanistically elucidated, because the cascade of variables linking BN and Ac contained degraded NDF and Val as intermediates. These matches with biological expectation support the strength of both network analyses for exploring complex data such as rumen fermentation variables. Another possible explanation for inverse associations between Ac and each of NAN, urea, or NO_3^- is, the possible toxicity of NO_2 to some microbes, particularly methanogens or may be due to an insufficient adaptation period for NO_3-supplementation (Guo et al., 2009). The interaction between NO_3- and Ac also depends on NO_3- reducer's lower affinity for aqueous H2, and so whether Ac decreases with NO_3- probably depends on if the increased aqueous H2 thermodynamically decreases Ac production. Additionally, Ac is commonly taken up by many bacteria that produce butyrate

(Wenner et al., 2020). Although the literature from continuous culture fermenters on Ac and N relationships is limited, studies reporting on VFA concentrations and N sources within in vivo studies have mixed results. Li et al. (2012) reported that either total VFA concentrations or individual VFA concentrations were not affected by the dietary N source in sheep. Those authors observed that, compared to urea, inclusion of nitrate increased concentrations of Ac, which is contradictory to network depiction and previous continuous culture experiments. Olijhoek et al. (2016) reported no significant relationship between dietary NO_3^- and VFA concentrations in dairy cows. A significant positive relationship between dietary NO_3^- and both total VFA and Ac was observed by Zhao et al. (2015) in steers fed different levels of NO_3. The network analysis approach provides some opportunity to make sense of these conflicting data from the literature by better evaluating concurrent fermentation shifts which may help explain the differences in responses observed previously (Wenner et al., 2020).

Both networks also identified important associations among Ac, IBu, and Val, suggesting integration between major and minor VFA in the continuous culture system. The BLN selected Val as a driving variable affecting Pr, Ac, IBu and IVa. These associations have been supported by some previous work. For example, Kristensen et al. (2000) reported increased Val concentrations with increasing Bu replacing Ac infusion. In a continuous culture study by Kone et al. (1989) in which the base diet was supplemented with branched chain VFA (BCVFA) and Val, increased Ac concentration was reported. Both findings corroborate the role of Val in influencing both major and minor VFA profiles. The strong association of Val with Ac and Pr can also be attributed to the fact that Val is a condensation of Pr and Ac (Wiltrout and Satter 1972) or potential usage of Ac-CoA to produce Val. French et al. (2012) observed that Val concentration was greatest during Pr infusion, and these align with biological expectations supporting relationships observed in both networks. Although there is growing interest for studies on the effect of BCVFA on rumen fermentation, studies on Val are limited. For that reason, a considerable number of interactions of Val with other variables we observed in both networks are not explicitly backed by existing literature. Because Val does not directly fall under the definition of BCVFA, it is not completely accurate to discuss associations of Val using these studies as references. A previous study reported that Val has no significant effect on NDFd and (Roman-Garcia et al., 2021) and is extensively produced from branched chain amino acids (BCAA). Given this finding and based on our network observations we can lead the discussion of Val into two directions. Since we did not include BCAA data to establish the network structure, it is possible that algorithm chose the next potential relationships, and Val is a proxy for BCAA within the network. Alternatively, Val may have an impact on rumen fermentation parameters which are yet to be explored extensively suggesting the necessity of future studies specifically exploring the role of Val with respect to rumen VFA stoichiometry.

Although Ac was identified as the primary intermediate between VFA and N-related variables, other VFA were mainly associated with one another. Within the ELN network, these associations manifested as a cluster of VFA nodes with strong positive associations. Within the BLN, the quantitative association manifested as a cascade of VFA nodes driven by Va and Bu. The strong positive associations among these variables are possibly suggestive of the role of interconversions among VFA or their common metabolic precursor, pyruvate, within microbial fermentation systems. Previous isotope-based studies reported that interconversions accounted for 14 to 17 % of Ac net production, 13 to 18 % of Pr net production, and 58 to 68 % of Bu net production (Sutton et al., 2003). Similarly, Ghimire et al. (2017) reported a minimal conversion of Pr to Bu and higher conversion of Bu to Pr of 31 % and 93 % of de novo Bu production, respectively in normal and high concentrate diets. This result was validated in another study (Li et al., 2022) and in these studies, Bu resulted in greater interconversion rates than did the other major VFA, which may support its identification as a driving variable within this VFA cascade. Because interconversions are thought to be influenced by microbial communities responding to rumen thermodynamic conditions (Kohn and Boston, 2000), future work should focus more explicitly on training networks with better representation of those thermodynamic conditions. Further studies on associations among microbial communities and interconversions of VFA would permit better understanding of the role of these factors in dictating the VFA profile.

Nitrogen Flows

The networks differed in terms of the roles they identified for N sources. Although both networks identified quantitative associations among urea and NO_3^-, they differed in terms of how they treated NH_3-N, NAN, and BN. In the ELN networks, NH_3-N, NAN, and to a lesser extent, BN, were highly central nodes within the network, providing linkages among VFA and degradability variables. In the BLN, BN, NO_3^-, NH_3-N, and urea were identified as drivers of the network dynamics, influencing factors like NDFd and VFA concentrations. That said, NAN was identified as a responding factor, driven by shifts in Ac, Bu, and BN. Traditional understanding of N flows within the rumen would suggest BN is a function of N and carbohydrate supplies (Roman-Garcia et al., 2016), and RN is predominantly associated with the type of intake N consumed within the diet (White et al., 2017). These networks suggest slightly more nuanced quantitative associations among N fractions and fermentation variables. For example, the driving role of BN identified within the BLN highlights the importance of considering the real feedback loops inherent within the rumen environment (which are better exemplified within the ELN network) because the data suggest BN has a driving role in controlling rumen fermentation; however, our understanding of the rumen environment suggests BN is both responding to and modifying fermentation patterns (which is supported by the centrality within the ELN). These feedback mechanisms are important to include in networks of complex biological systems; thus, there is some advantage to use of the ELN strategy. In this case, greater degradability of carbohydrate increases the production of both VFA and microbial matter, but it is influenced by how carbon is routed either to VFA or microbial matter (Hackmann and Firkins 2015, Firkins, 2021). If the efficiency of microbial protein is improved, a greater percentage of carbon will route towards microbial growth and a lower percentage toward VFA (Russell, 1995). In the present comparison, the ELN better encapsulates these associations than the BLN. The strength of the ELN is not consistent, however. For example, the central role of NAN identified in both networks is somewhat challenging to reconcile with the other N flows and merits further investigation. These discrepancies in the way the networks represent rumen N dynamics might reflect the differences between animal studies and fermentation studies or could alternatively suggest that additional work focused on characterizing the causal or responsive nature of N fractionation within the rumen is imperative to improving understanding of factors controlling the fermentation environment. Further investigation of the associations among VFA and ruminal N variables are warranted to better evaluate how these factors influence efficiency of fermentation.

Intake and Nutrient Degradability

Feeding rate (i.e., DMI) in networks was positively associated with Bu, Val, Pr, and OMd and negatively associated with IVa, Ac, and NH_3-N concentrations in the ELN (Fig. 1). In the BLN (Fig. 2), DMI was an intermediate variable, driven by Val and NH_3-N and influencing Ac and OMd. Within the dataset used for network determination, DMI was an experimentally defined variable and interpretation of DMI associations should reflect only the causal influence of adding additional substrate into the fermentation, rather than inferring information about feed intake regulation. As such, the associations revealed within the ELN model suggest that, as additional substrate is provided, we can expect higher OMd and greater concentrations of some VFA in CC because the passage rate is not increased as would passage rate increase with increasing DMI in vivo. Shifting to fermentations with greater available substrate resulted in less Ac, reduced NH_3-N, and higher concentrations of Pr, Val, and Bu. The changes in fermentation caused by substrate availability revealed within the ELN are useful in modifying rumen fermentation towards Pr production which suppresses CH_4 production.

In both networks, there was considerable agreement regarding how NDFd and OMd should be incorporated into the picture of fermentation. Within the ELN, NDFd was positively associated with OMd and NH_3 and negatively associated with BN. In the BLN, the same NDFd associations with BN and NH_3-N were observed (as influencing factors), and the association with OMd was highlighted as a response to shifting NDFd. More microbial growth means more NH_3-N assimilated into microbial N, meaning that more degradability results in more microbial N (Pengpeng and Tan, 2013). Therefore, the associations among BN, NH_3-N, and NDFd within the network are well explained by current understanding of factors governing fermentation. Similarly, OMd was identified within the BLN as influenced by FF, Pr, NDFd, and DMI. Previous models of OMd and NDFd highlight protein availability and degradability in the rumen, as well as rumen pH, as critical variables influencing the extent of fiber and organic matter degradation within the rumen. Griswold et al. (2003) observed the supplementation of rumen degradable protein (urea in the current work) increased all degradability of OM and NDF ($P < 0.05$) when added to a protein-limiting diet. The enhanced protein degradability enabled increased NH_3-N and subsequently microbial protein synthesis. These relationships identified in previous research partially agree with the positive quantitative association between the above variables depicted in both networks derived herein. Broderick and Reynal (2009) also observed linear increases in rumen NH_3 concentration and NDFd with urea supplementation. These associations are likely most relevant to moderate to high quality forages because in a study feeding very low quality forage, Sawyer et al., (2012) did not find an effect on NDFd when supplementing a cattle diet with urea, which suggests that in very low quality forage NDFd may be more limited by forage lignification, rather than N availability. These observations from previous studies concur with the associations identified in the networks and suggest that N related variables can have a major influence on the degradability of fiber and organic matter when the quality of fiber source is high.

Other Fermentation Variables

Rumen protozoa have been identified as key players in the rumen, helping to facilitate nitrogen recycling through degradation of rumen bacteria (Firkins et al., 2020) and CH_4 production (Newbold et al., 2015). In the ELN, the association between protozoa and CH_4 is direct and positive, which follows current assumptions that defaunation results in depressed CH_4 emissions (Martin et al., 2010; Nguyen and Hegarty, 2016). That said, at least a significant part of that relationship must be associated with improved fiber degradability and resulting dihydrogen formation (Firkins, 2021). Removal of protozoa can also be detrimental for fiber digestion because defaunation can cause a drop in fibrolytic bacteria in the rumen (Newbold et al., 2015). However, in the BLN, we are better able to infer mechanistic responses, in which PZ and CH_4 are modulated by BN. Although this association is not revealed by a proper feedback loop, as we might expect given our understanding of rumen fermentation, the nature of the DAG prevents feedback loops, and therefore the dynamics of the system must be inferred from the flowthrough of causal quantitative associations identified. This limitation of the BLN is an important caveat for considering their use in interpreting the rumen environment moving forward. Although the quantitative associations identified within the BLN are clearer and more concise, they may oversimplify implied causality by leaving the reader to infer and leverage feedback mechanisms known to be influential in the regulation of biological and ecological processes to make sense of the identified network structure.

Limitations

As a preliminary investigation of the opportunity to leverage network analysis for improved understanding of rumen fermentation parameters, this study has a number of limitations. First, the data used for exploration were generated from a single lab due to availability and the goal of minimizing between-study variability. Although this contributed to a relatively clean dataset for comparison of the different networking approaches, the breadth and broader interpretability of the inferences drawn from the data are limited. Second, we analyzed the data as a uniform set, rather than explicitly controlling for between-study variation. Although there are network approaches which support between-study variation, the authors are only aware of a Bayesian approach which explicitly treats this between-study variation under a random variable paradigm, as would be common practice in a meta-analysis. As such, explicitly considering between-study variation would have precluded comparison of the frequentist and Bayesian networking approaches, which was the primary goal of this work. Nevertheless, it is possible that the networks have mis-apportioned variation that should be attributable to common differences among these 04 studies to measured values. As such, the results are most applicable to preliminary exploration of the differences among these methods in defining relationships and variables of interest within a complex and interrelated environment such as the rumen, rather than for making explicit inference about the biological responses and associations observed. That said, the overarching coherence between the relationships revealed as important by the networks and our general understanding of rumen fermentation provide confidence in the strength of the dataset and the approaches for gleaning improved holistic understanding of this complex ecosystem.

CONCLUSIONS

The different networking approaches provided complementary opportunities to explore complex associations inherent within the rumen environment. The ELN approach generated an undirected network showing feedback loops whereas the BLN was a DAG showing implied causality of associations. Therefore, it is fair to say that BLN is relatively the stronger network analytical approach to visualize and understand control pathways within complex ecosystems. The frequentist network, however, allowed for more explicit exploration of the types of relationships held by different nodes and the greater flexibility of this approach allowed for feedback loops to occur within the network structure, as they are known to occur within biological systems. As such, the ELN has the primary advantage in highlighting nodes of interest within complex ecosystems, and important feedback mechanisms in those systems. However, the strength and the stability of both networks were mediocre, likely due to the smaller sample size and the variability of fermentation measurements. Despite these differences and limitations, the networks could collectively be leveraged to draw biological inferences relevant to understanding factors controlling fermentation. Other relationships identified highlighted opportunities for future work. Particularly, the role of valerate within both networks suggested future work to refine the role of valerate in fermentation dynamics may be beneficial. Similarly, future work exploring how N fractions contribute to different fermentation characteristics may be warranted. For many nodes, the different network approaches were complementary, allowing for more robust evaluation of mechanism and relationships. Collectively, the results suggest that network analyses, and specifically studies leveraging multiple approaches to network analysis, may be a supporting tool helpful in better interpreting and learning from complex experimental datasets, such as those describing the rumen environment. Despite this opportunity, limitations in the strength and interpretation of the different approaches to network construction should be carefully considered when drawing biological inference.

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Tables

Table 4-1. Key features of the four experiments considered in this network analysis.

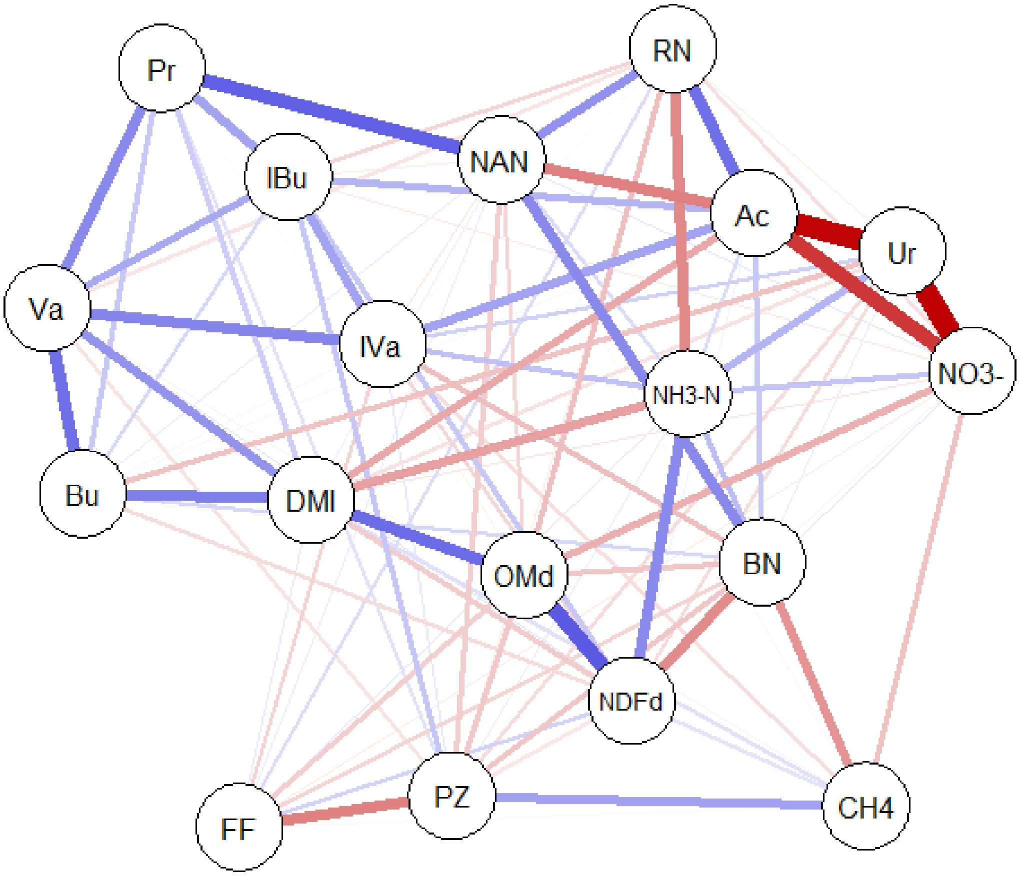
Experiment	**1**	**2**	**3**	**4**
Reference	Wenner et al., 2017	Wagner et al., 2018	Welty et al., 2019	Wenner et al., 2020
DM Fed, g/d	36.8	60.0	36.3	40.0
Feed Frequency	1x/d	2x/d	1x/d	1x/d
Design	Latin square	Latin square	Youden square	Latin square
Treatments	pH, kp	CP, starch sources	Yeast, nitrate	Nitrate, defaunation
Solids k_p	2.5% vs 5.0%	2.5%	2.5%	2.5%

Table 4-2. Arc strength and accuracy of predictability of individual nodes in Bayesian learning network.

Arc strength and direction			**Accuracy of predictability**		
From	**To**	**Strength**	**Direction**	**Node**	**CCC**
Ac	NAN	0.910	0.755	Ac	0.97
Ac	RN	0.905	0.566	Pr	0.96
Ac	Ur	0.995	0.979	IBu	0.93
Val	Pr	0.925	0.702	Bu	0.96
NAN	Pr	0.950	0.428	IVa	0.80
Val	IBu	0.915	0.934	Val	0.98
Bu	Val	0.960	0.729	FF	0.39
Val	Pr	0.925	0.702	NH_3-N	0.55
Val	IBu	0.915	0.934	NAN	0.90
BN	NAN	0.920	0.608	OMd	0.87
DMI	OMd	0.975	0.717	NDFd	0.74
Ac	IBu	0.775	0.780	CH_4	0.58
Val	DMI	0.760	0.944	RN	0.61
NDFd	OMd	0.760	0.812	BN	0.77
BN	CH_4	0.825	0.327	PZ	0.63
NH_3-N	RN	0.885	0.474	DMI	0.99
BN	PZ	0.705	0.688	NO_3^-	0.93
Ac	Val	0.165	0.606	Ur	0.96

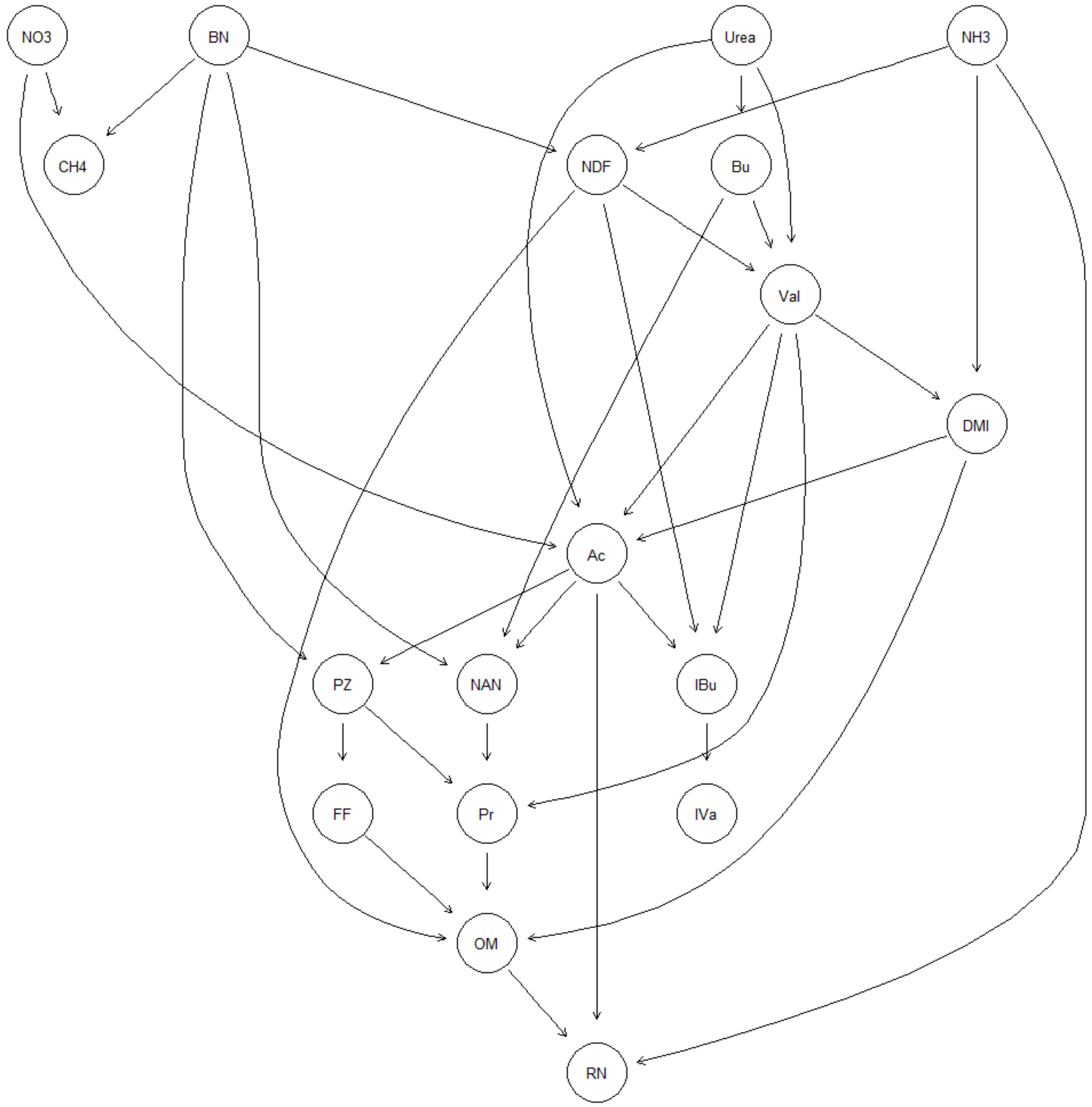
Strength and the direction of selected arcs are presented, and values ranged between 0 to 1. Higher the value for strength higher the strength of the arc. Value for direction indicates the potential direction; values above 0.5 denotes the exact direction as listed From to To in the table while values below 0.5 denotes possibility of the reverse direction of the arc. Accuracy of estimating each node is presented with Lin's Concordance Correlation (CCC) value where higher value indicates higher accuracy in prediction. Ac (acetate, m*M)*; Bu (butyrate, m*M)*; Pr (propionate, m*M*); Val (valerate, m*M*); IBu (isobutyrate, m*M*); IVa (isovalerate, m*M*); DMI (dry matter intake, g/d); OMd (organic matter degradability, %); NDFd (neutral detergent fiber degradability, %); FF (fluid flow, L/d); CH_4 (methane, mmol/d); PZ (protozoa, cells/ml); NAN (non-ammonia nitrogen, g/d); NH_3-N (ammonia nitrogen, mg/dL); BN (bacterial nitrogen, g/d); NO_3- (nitrate, %); RN (residual N, g/d)

Figure 4-1. EBIC-LASSO network depicting the quantitative association among rumen fermentation variables.



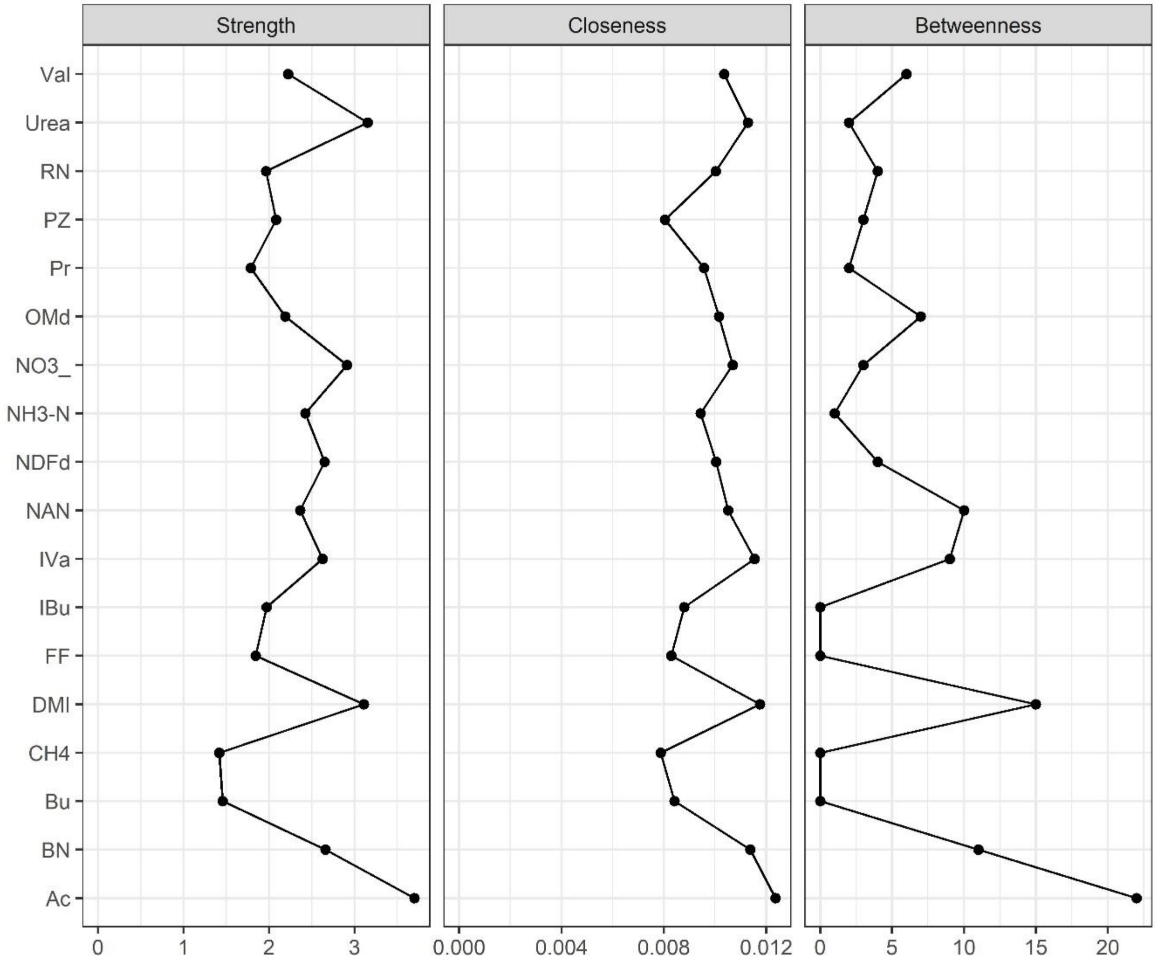
Variables are represented by nodes and quantitative association among variables are represented with edges. Blue and red color edges denote the positive and negative quantitative association, respectively. Thickness of the edge represents the strength of the relationship. Ac (acetate, mM); Bu (butyrate, mM); Pr (propionate, mM); Val (valerate, mM); IBu (isobutyrate, mM); IVa (isovalerate, mM); DMI (dry matter intake, g/d); OMd (organic matter degradability, %); NDFd (neutral detergent fiber degradability, %); FF (fluid flow, L/d); CH_4 (methane, mmol/d); PZ (protozoa, cells/ml); NAN (non-ammonia nitrogen, g/d); NH_3-N (ammonia nitrogen, mg/dL); BN (bacterial nitrogen, g/d); NO_3- (nitrate, %); RN (residual N, g/d)

Figure 4-2. Bayesian learning network of rumen fermentation variables.



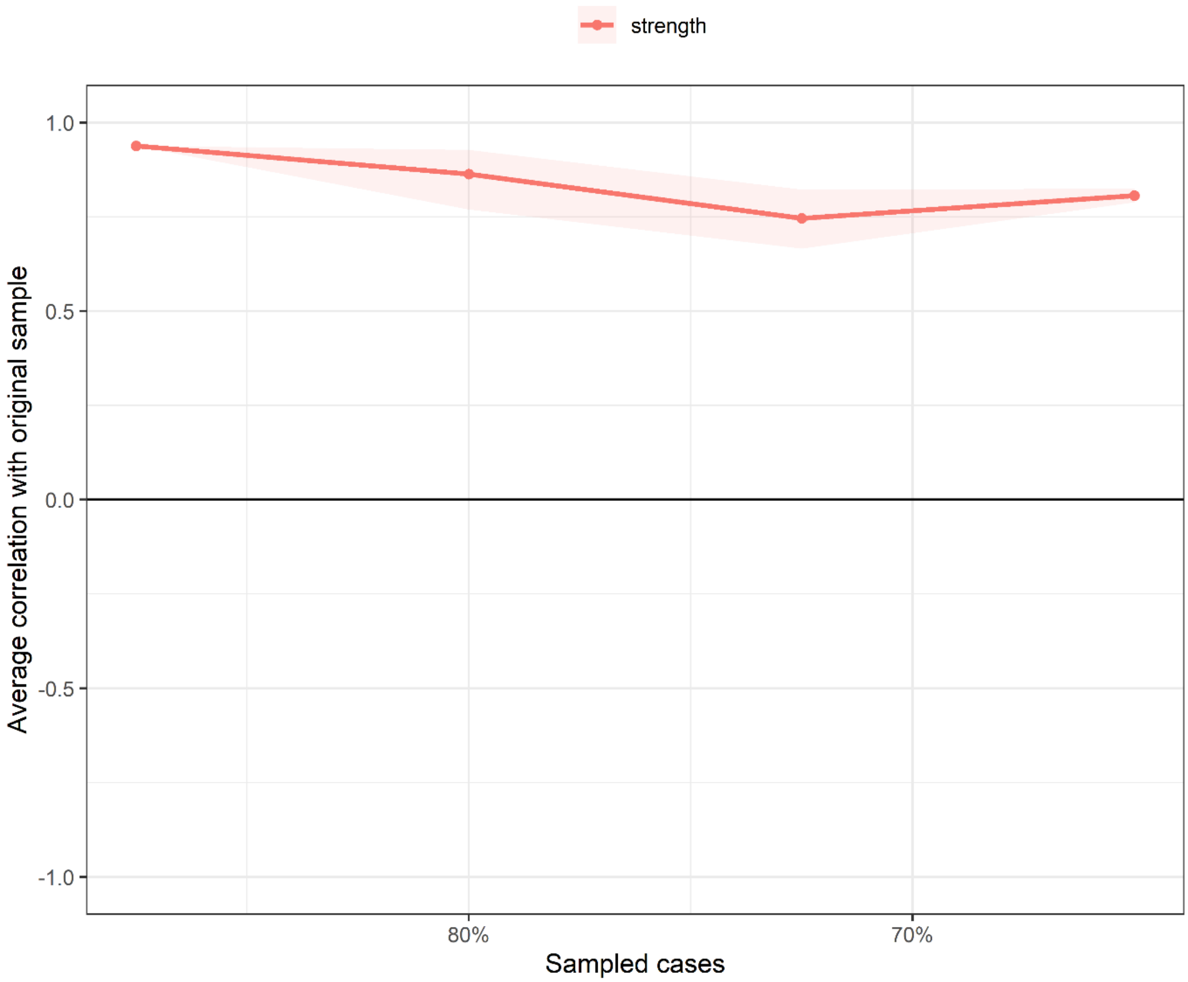
Variables are represented by nodes and quantitative association among variables are represented with edges. Directionality of the edge indicates the direction of the relationship among two variables. Bayesian learning network is a DAG with a cascading nature hence no feedback loops among nodes. Ac (acetate, mM); Bu (butyrate, mM); Pr (propionate, mM); Val (valerate, mM); IBu (isobutyrate, mM); IVa (isovalerate, mM); DMI (dry matter intake, g/d); OMd (organic matter degradability, %); NDFd (neutral detergent fiber degradability, %); FF (fluid flow, L/d); CH_4 (methane, mmol/d); PZ (protozoa, cells/ml); NAN (non-ammonia nitrogen, g/d); NH_3-N (ammonia nitrogen, mg/dL); BN (bacterial nitrogen, g/d); NO_3- (nitrate, %); RN (residual N, g/d)

Figure 4-3. Corresponding centrality indices of the constructed network.



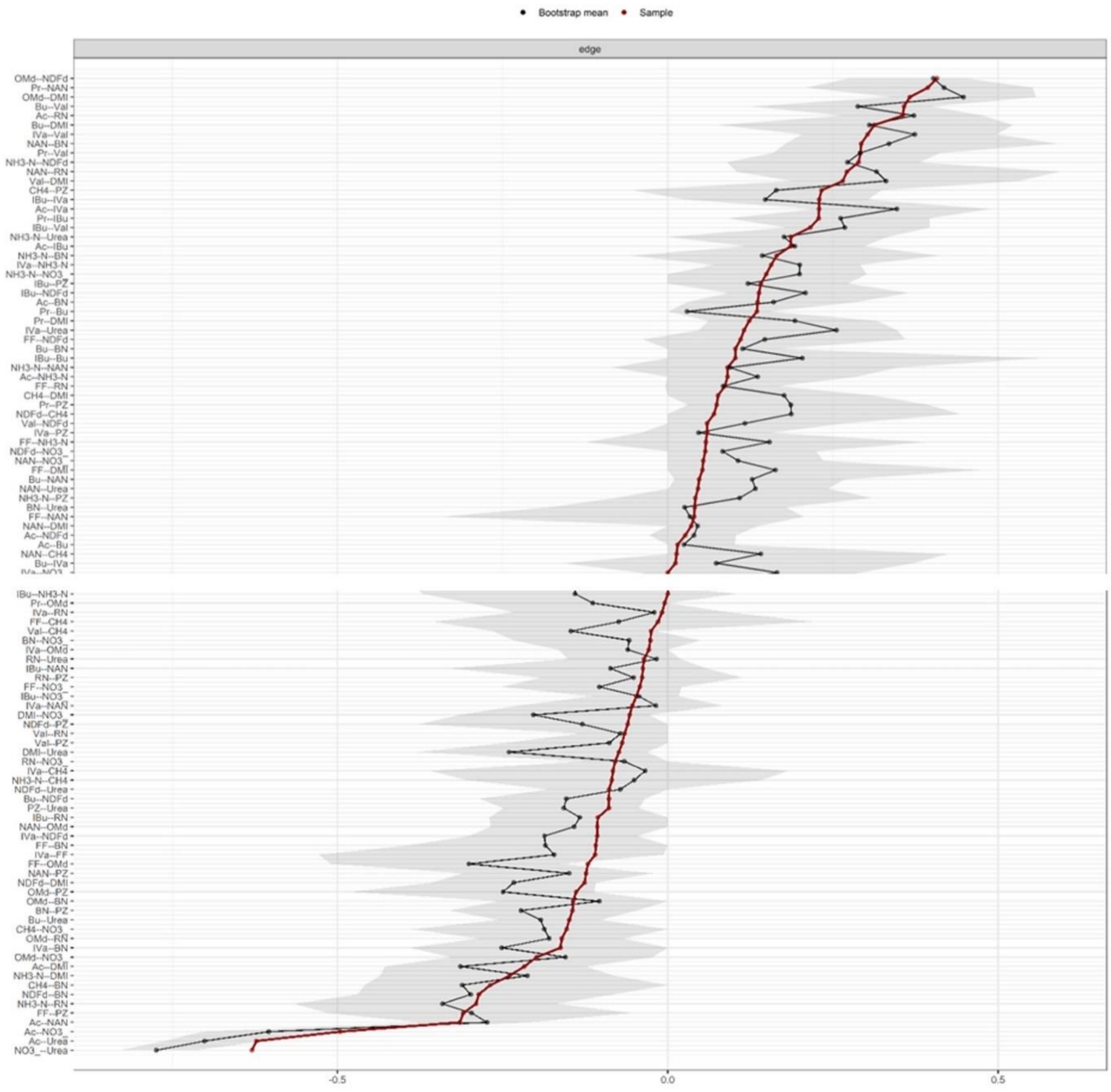
Ac (acetate, mM); Bu (butyrate, mM); Pr (propionate, mM); Val (valerate, mM); IBu (isobutyrate, mM); IVa (isovalerate, mM); DMI (dry matter intake, g/d); OMd (organic matter degradability, %); NDFd (neutral detergent fiber degradability, %); FF (fluid flow, L/d); CH_4 (methane, mmol/d); PZ (protozoa, cells/ml); NAN (non-ammonia nitrogen, g/d); NH_3-N (ammonia nitrogen, mg/dL); BN (bacterial nitrogen, g/d); NO_3- (nitrate, %); RN (residual N, g/d)

Figure 4-4. Average correlations of strength centrality of the sampled cases.



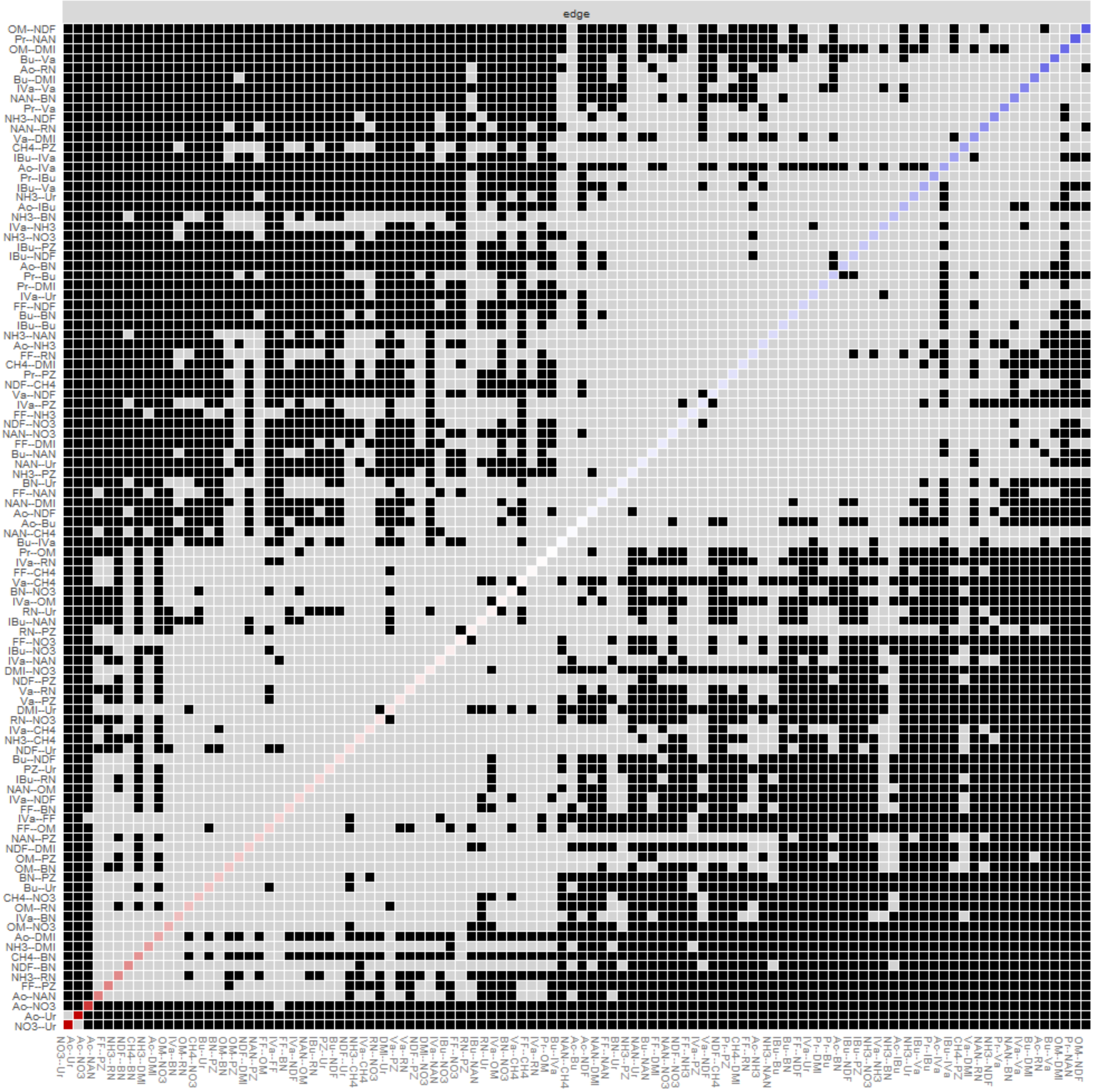
X axis shows the percentage of sampled cases against average correlation with the original sample in the y axis.

Figure 4-5. Bootstrapped confidence intervals of estimated edge-weights for the estimated network of rumen fermentation variables.



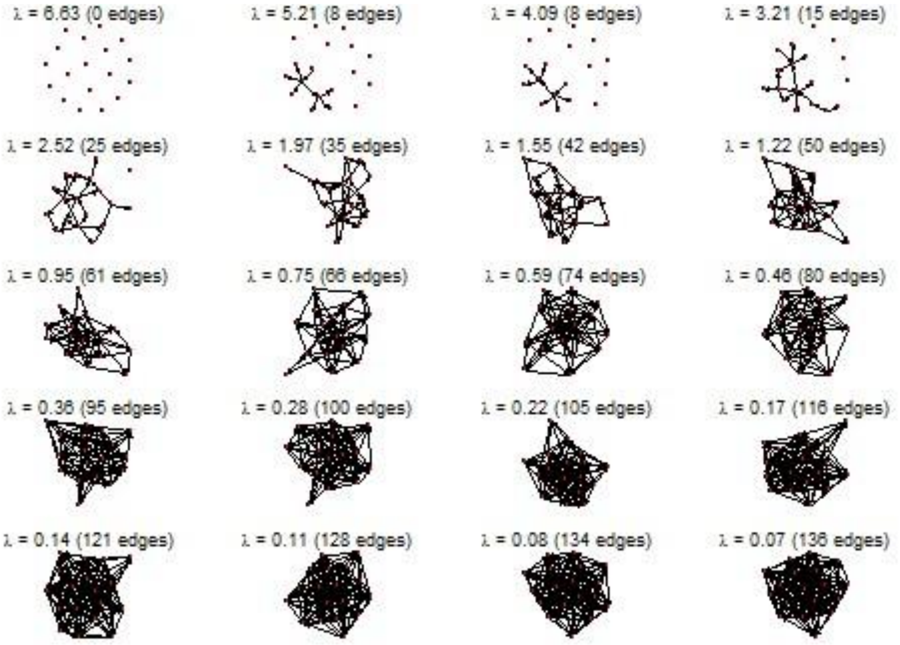
The Y axis denotes all the edges, and the X axis shows the strength of the edge weights. The red dots are the point estimates of all edges, and the gray area is the bootstrapped confidence intervals at 95%. Each horizontal line represents one edge of the network which is ordered from the highest edge-weight to the lowest edge-weight. Highest positive edge weights show strong positive quantitative association, but highest negative edge weights represent strong negative quantitative association among variables. Only the edges with positive and negative values are extracted from the edge weight graph for the sake of clarity and readability of the graph. Ac (acetate, mM); Bu (butyrate, mM); Pr (propionate, mM); Val (valerate, mM); IBu (isobutyrate, mM); IVa (isovalerate, mM); DMI (dry matter intake, g/d); OMd (organic matter degradability, %); NDFd (neutral detergent fiber degradability, %); FF (fluid flow, L/d); CH_4 (methane, mmol/d); PZ (protozoa, cells/ml); NAN (non-ammonia nitrogen, g/d); NH_3-N (ammonia nitrogen, mg/dL); BN (bacterial nitrogen, g/d); NO_3^- (nitrate, %); RN (residual N, g/d)

Figure 4-6. Bootstrapped difference tests ($\alpha = 0.05$) for edge weights that were non-zero in the estimated network.

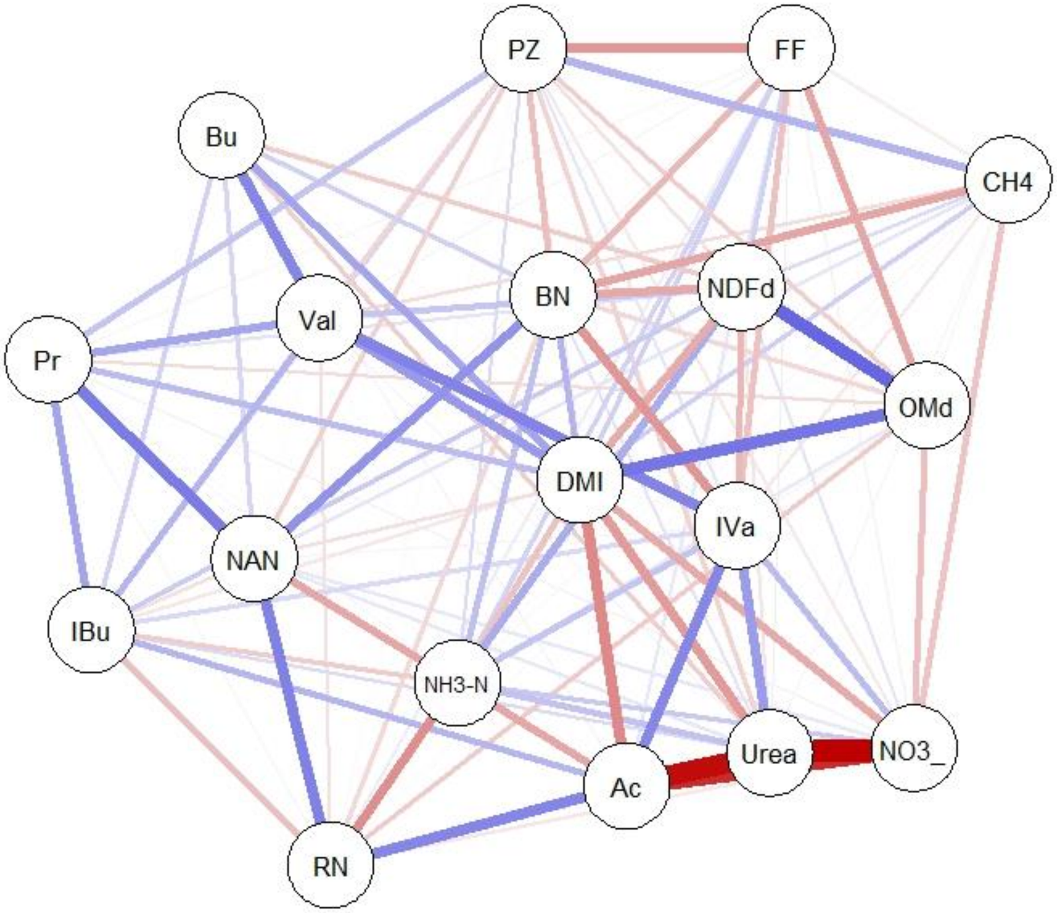


Axes display all possible edges (i.e., (n*(n-1))/2), with n being the number of nodes considered). Color of the box denotes the significance between edge-weights where black boxes indicate significantly different edge-weights from each other, and grey boxes indicate edge-weights which are not significantly differ in between. The diagonal line indicates the strength of edge-weights, shifting from red (negative associations) to white (representing weaker edges) and ultimately to blue (representing stronger edges aka positive quantitative association). Ac (acetate, mM); Bu (butyrate, mM); Pr (propionate, mM); Val (valerate, mM); IBu (isobutyrate, mM); IVa (isovalerate, mM); DMI (dry matter intake, g/d); OMd (organic matter degradability, %); NDFd (neutral detergent fiber degradability, %); FF (fluid flow, L/d); CH_4 (methane, mmol/d); PZ (protozoa, cells/ml); NAN (non-ammonia nitrogen, g/d); NH_3-N (ammonia nitrogen, mg/dL); BN (bacterial nitrogen, g/d); NO_3- (nitrate, %); RN (residual N, g/d)

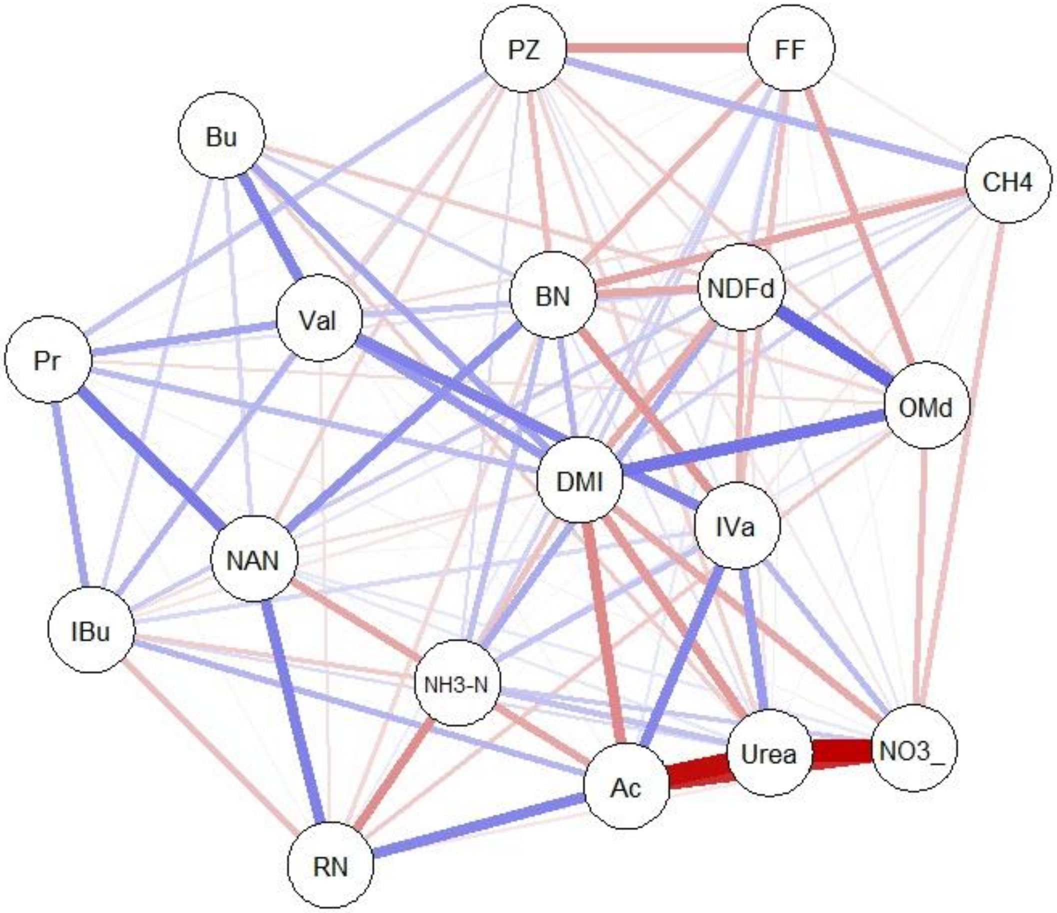
Supplementary Figure 1. EBIC-LASSO networks associated with different lambda values.



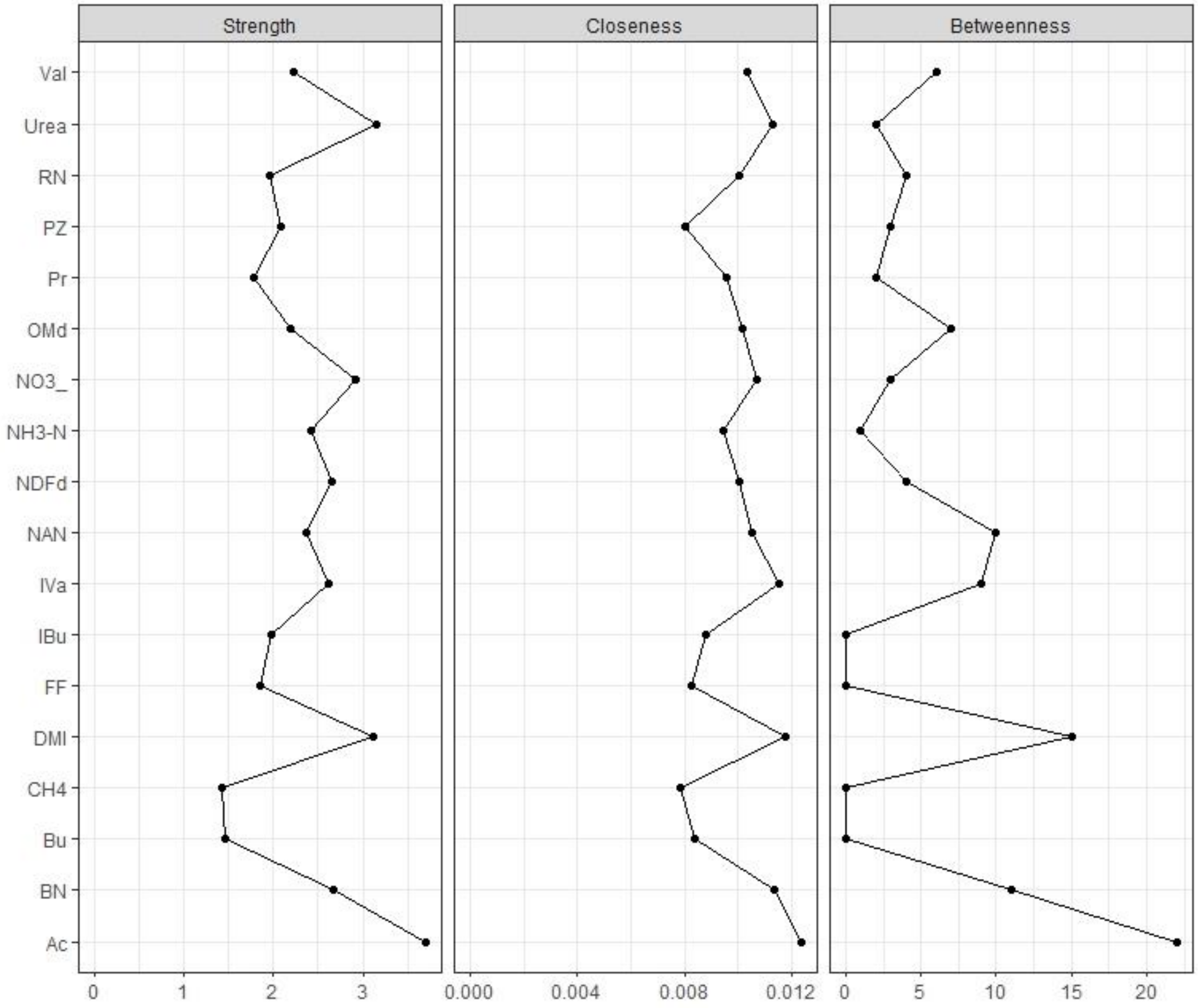
Supplementary Figure 2. EBIC-LASSO network associated with lambda value of 0.75 depicting the quantitative association among rumen fermentation variables.



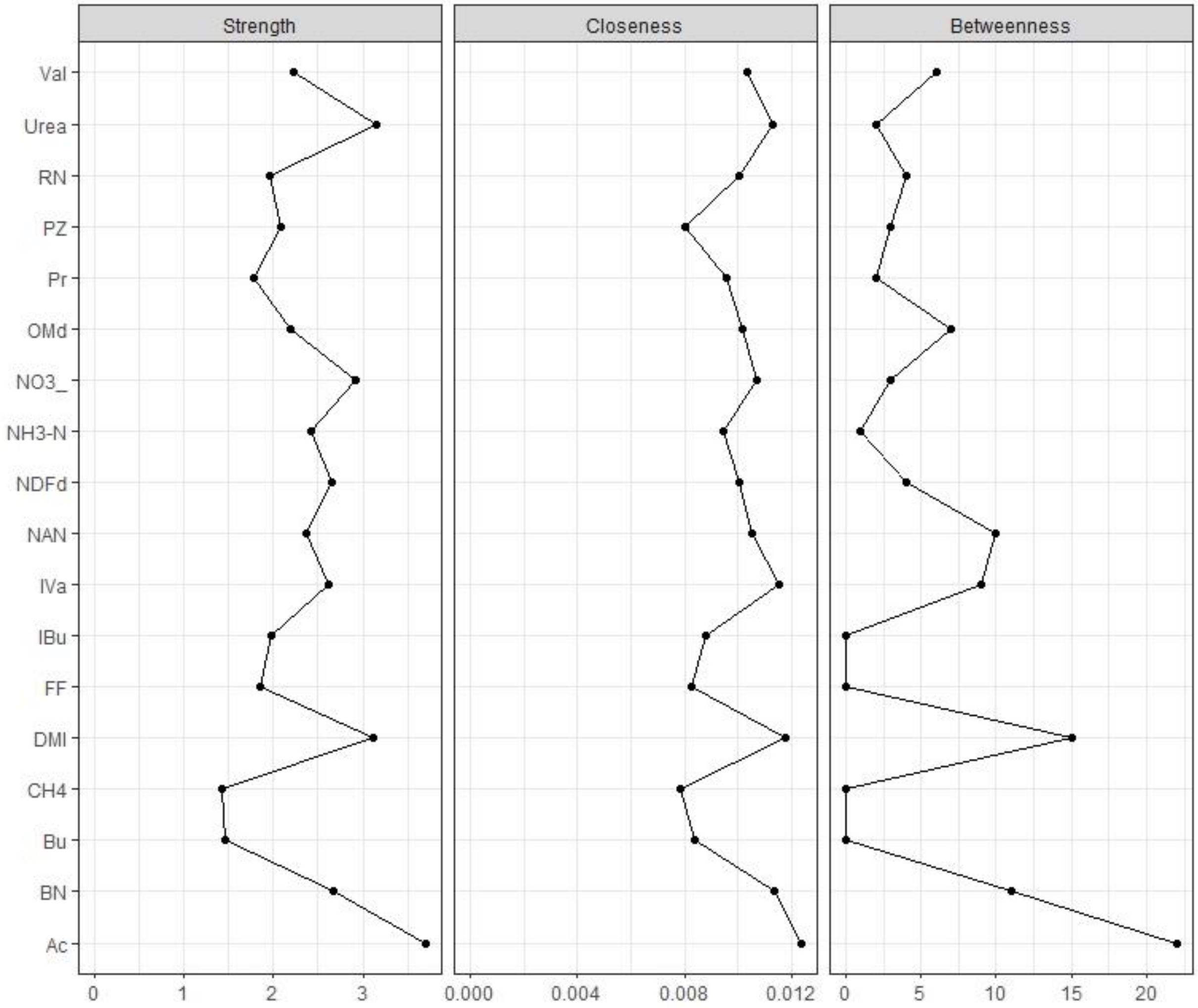
Supplementary Figure 3. EBIC-LASSO network associated with lambda value of 0.46 depicting the quantitative association among rumen fermentation variables.



Supplementary Figure 4. Corresponding centrality indices of the network of lambda 0.75.



Supplementary Figure 5. Corresponding centrality indices of the network of lambda 0.46.



Chapter 5 : Rumen Fermentation of Meal Fed Sheep in Response to Diets Formulated to Vary in Fiber and Protein Degradability

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This chapter will be submitted as S. Sujani, C.B. Gleason, B.R dos Reis and R.R White. Rumen fermentation of meal fed sheep in response to diets formulated to vary in fiber and protein degradability, Journal of Animal Science, 2023.

ABSTRACT

Although widely used, VFA concentrations provide an imprecise view of VFA supplies to animals due to the confounding effects of fluid pool size and dynamics. Alternatives such as VFA flux determination using isotope are available; however, these are expensive and sensitive methodologies which require animals to maintain a metabolic steady state which may not accurately represent the rumen environment during normal feeding conditions. In light of these challenges, a more rapid, affordable, and physiologically relevant approach to exploring VFA dynamics may allow broader and more economical characterization of VFA availability generated by different diets. The objective of this study was to explore the use of VFA dynamics generated by meal feeding to derive time-series rates of VFA apparent appearance and disappearance driven by diets differing in protein and fiber sources. Six ruminally cannulated wethers were fed diets containing timothy hay or beet pulp (TH, BP) and soybean meal (SBM) or heated soybean meal (HSBM) in a 2x2 factorial arrangement of treatments (TH + HSBM; TH + SBM; BP + HSBM; and BP + SBM). The experimental design was a partially replicated 4 × 4 Latin Square (n=6). Rumen fluid samples were analyzed for VFA and polyethylene glycol (PEG) concentrations. Concentrations of PEG were used to estimate fluid passage and volume to calculate VFA mass, and fluid-mediated exit. Maximum apparent appearance rate (mmol/h), the rate of apparent appearance decline (mmol/mmol/h), mean apparent appearance flux (mmol/h), mean apparent disappearance (mmol/h) and apparent disappearance rate (mmol/mmol/h) were estimated by deriving a 1 pool model for each VFA on a mass basis where appearance was assumed to follow an exponential decay pattern and disappearance followed mass action kinetics. Statistical analyses on rates and fluxes were conducted using a linear mixed effect regression with fixed effects for fiber source, protein source, and their interaction, as well as random effects for animal and period. Dry matter intake and rumen pH were unaffected by protein and fiber sources. Rumen fluid volume (L) was greater in HSBM diets (P = 0.033) and fluid passage (%/h) was greater in SBM diets (P = 0.048). Concentrations and molar proportions of VFA were affected only by fiber source; however, when viewed under the kinetics lens, protein source and fiber source interacted to significantly influence apparent appearance rates and absorption rates of many major VFA. On a flux basis, protein source (HSBM) supported significantly elevates estimates of apparent disappearance. These data demonstrate that time-series evaluation of fermentation dynamics provides an opportunity to integrate information from fluid dynamics and VFA concentrations to provide estimates of apparent appearance and disappearance of VFA. Although further work is needed to confirm the alignment of these estimates with measurements of VFA supplies to the animal, this simple modelling approach provides minimally a way to better understand the kinetics of fermentation occurring within the rumen.

Key words: Beet pulp, iso-acids, passage rate, time-series evaluation, volatile fatty acid dynamics

INTRODUCTION

Volatile fatty acids (VFA) are the most important fermentation end products of ruminants and provide approximately 70 % of the total energy requirement to fuel productive functions of the host animal (Bergman et al., 1965; Bergman, 1990). Type of substrate fermented, microbial population, and rumen environment influence the type of VFA synthesized in the rumen (Bannink et al., 2008), and different VFA are used with differing physiological efficiencies for various metabolic processes (Baldwin, 1995). The majority of rumen fermentation studies have focused on VFA concentration which are not reliable estimators of VFA supplies to the animal because they do not always represent synthesis, interconversion, absorption, and passage of VFA, or the volume of rumen fluid (Hall et al., 2015). Nevertheless, accurate estimations of VFA dynamics are vital to illustrate ruminal function and ruminal efficiency.

A variety of techniques have been used to estimate production and absorption rates of VFA from the rumen. The techniques to estimate VFA production in the rumen can be categorized as tracer based and non-tracer based methods. Some of the widely based non-tracer based methods are: incubation and fermentation of rumen contents in vitro (Tilley and Terry, 1963; Menke et al., 1979), measurements of net VFA absorption by analysis of arterial and portal blood together with measurements of rates of portal blood flow (Bergman and Wolff, 1971), and rumen bail out method (Reid et al., 1967; Kristensen and Harmon, 2004). Under tracer-based methods isotope-based assessments of rumen VFA offer more accurate means of measuring VAF dynamics (Makkar, 2008; Nolan et al., 2014) because these experiments allow for estimation of actual synthesis, interconversion and absorption of VFA. Given the improved computational power isotope based data can be used to calculate these VFA fluxes. Continuous intraruminal infusions of VFA isotope has been successfully utilized by previous studies (Esdale et al., 1968; Bedford et al., 2020; Gleason et al., 2022b) to estimate VFA synthesis, interconversion and absorption. However, these studies are expensive, labor-intensive, and mostly rely on feeding strategies to induce a metabolic steady state which may not reflect the normal physiological state (Hegarty and Nolan, 2007). Therefore, a less resource demanding method relative to the isotope based approaches to estimate VFA dynamics under non-steady state (representing the commonly practiced feeding regiments) may greatly benefit future research on rumen fermentation.

As an alternative to the isotope based assessment, we suggest evaluation of a time-series of fermentation indicators (Krämer et al., 2013) with regular meal-feeding of animals may be a lower-cost, more efficient screening approach to compare fermentation conditions. In this approach, the meal is treated as a bolus of substrate from which the net appearance of VFA is driven. Appearance follows an exponential decay pattern of a marker concentration (e.g., polyethylene glycol) as described in a study by Beckett et al. (2021) and can be used to reflect the summation of de novo synthesis and interconversion from other VFA. Similarly, net apparent disappearance reflects the summation of absorption and interconversion to other VFA. Explicitly, with this screening method we will not estimate the production and absorption of VFA but net appearance and net disappearance. Due to the physiological differences in the suggested method, the major interests were to explore the ability to derive rates from fermentation curves, and in evaluating the biological relevance of the rates estimated. With the aid of improved power of computation and more complicated modelling approaches, it may also be possible to leverage feeding dynamics as a metric of VFA dynamics. Comparisons to other methods of VFA flux determination were out of scope due to the challenges of comparing meal-fed animals with those maintained artificially in a metabolic steady-state.

The majority of the previous studies which estimated ruminal VFA dynamics focused on the effect of different ratios of forage: concentrate and different feed sources rather than different rumen degradability of nutrients (Cantalapiedra-Hijar et al., 2009; Wang et al., 2020; Chen et al., 2021). Because differences in ruminal degradability of nutrients can alter the profile of fermentation end products (Beckett et al., 2021; Gleason et al., 2022a), we hypothesized that differences of ruminal nutrient degradability will alter rumen volume, passage rate and VFA dynamics. Therefore, the objective of this study was to integrate rumen fluid volume, fluid passage rate, and VFA concentrations to provide estimates of fermentation dynamics in response to diets differing in ruminal degradability of fiber and protein sources in meal-fed animals.

MATERIALS AND METHODOLOGY

Animals, Diets and Experimental Design

All procedures and animal use of this study were approved by the Virginia Tech Institutional Animal Care and Use Committee (Protocol #19-200). Six ruminally cannulated commercial wethers (Suffolk, Dorset or Suffolk x Dorset) were housed individually in pens (saw dust as the bedding material) at the Smithfield Farm, Virginia Tech, Blacksburg, VA for the duration of the experiment. Wethers were approximately 3.5 years of age and an average of 74.33 ± 13.33 kg body weight at the beginning of the experiment.

Four dietary treatments consisted of a combination of a protein source and a fiber source intended to have differing ruminal degradability. Regular soybean meal (SBM) and heat-treated soybean meal (HSBM) represented highly and lowly degradable protein sources within the rumen, respectively. These (Table 5-1) protein sources were pelleted along with alfalfa, corn, barley, wheat middlings, trace mineral salt, and a sheep vitamin-mineral premix. Timothy hay (TH) (approximately 8-10 cm particle length) was used as the lowly degradable fiber source while beet pulp (BP) pellets (diameter of 2 mm and length 10 mm) served as the highly degradable fiber. Four dietary treatments were prepared daily by combining the appropriate protein pellet with the appropriate fiber source as follows: a) highly degradable NDF and lowly degradable CP (BP + HSBM), b) lowly degradable NDF and lowly degradable CP (TH +

HSBM), c) highly degradable NDF and highly degradable CP (BP + SBM), and d) lowly degradable NDF and highly degradable CP (TH + SBM) (Table 5-1). The experiment was designed as a partially replicated 4x4 Latin square design where four dietary treatments were arranged in a 2x2 factorial. The experiment consisted of 4 periods with 25 days in each period. Animals were gradually adapted to their respective diet during the first four days by increasing daily allowance by 25% each day to achieve 100% feeding of the net ration by the 4th day. From day 4 to day 25 of each period, animals were fed with the experimental diet once daily at 2 % of body weight on a dry matter basis at 0900 hr. Refusals were collected daily throughout the experiment to calculate dry matter intake. Animals had access to ad libitum clean fresh water throughout the day.

Feed Analysis

Approximately 100 g (as-is) of protein pellets, beet pulp pellets and timothy hay were collected weekly, composited, and stored at -20°C until 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 percentage was determined by combustion in a muffle furnace (Sybron Thermolyne FA1730, Fisher Scientific, Waltham, MA) for 12 h at 500°C. Concentrations of NDF and ADF were determined using the Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY) following the methods described by (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.

Polyethylene Glycol (PEG) Administration and Rumen Fluid Sampling

Administration of PEG and sampling was performed from day 22 to 25 (n = 4 days) of each period. A bolus of PEG (average MW 8000; Fisher Scientific, Hampton, NH; 11.2 g PEG dissolved in 100 mL of water) was administered intraruminally via the rumen cannula at 0800 h. Baseline rumen fluid samples were collected immediately before administration of the PEG. Each animal was fitted with a rumen fluid sampling device consisting of tygon tubing threaded through bored holes in the cannula cap and into the rumen fluid. Each line terminated in a plastic mesh pot scrubber weighted with steel nuts. One sampling line was placed in the cranial portion of the rumen and the other in the caudal portion. Rumen fluid was drawn from these two sampling lines (approximately 10 ml from each line), aliquoted in three 6 mL glass vials, and frozen at -20°C until analysis. Collection of rumen fluid samples started at 0800 hr, prior to PEG blousing, and continued every 30 min until 1200 hr. Thereafter, rumen fluid samples were continued hourly until 2400 hr. During sampling of the first period, a rumen bolus pH sensor (T9 data logger, 20.7 mm diameter, 140 mm length and weighted 230 g, DASCOR, Escondido, CA) which was set up to record pH every 10 minutes was placed in the rumen of each animal. The sensor was removed after the first period and due to data failure rates, rumen pH in the other three periods were measured using a handheld portable pH meter (Oakton pH 50 Spear Waterproof Pocket pH Testr, Fisher Scientific, Hampton, NH). Measurements obtained from the handheld digital pH meter were based on fluid samples collected every 3 hrs intervals starting from 0900 hr.

Rumen fluid samples of individual animals were composited across days within a period and samples from hours 0, 3, 6, 9, 12, and 15 post-feeding were analyzed for concentrations of VFA. Analysis of VFA concentrations was conducted at The Ohio State University using gas chromatography. Concentration of total VFA and individual VFA per lamb were determined using a Hewlett-Packard 5890 series gas chromatograph (Agilent

Technologies, Santa Clara, CA) equipped with a glass packed column (23110-U, Sigma-Aldrich) with N_2 as a carrier gas (24 mL/min). The inlet was 150°C, the flame ionization detector was 180°C, and the oven temperature was 175°C. Each run was 18 min long to separate acetate (retention time: 1.6 min), propionate (3.0 min), isobutyrate (5.2 min), butyrate (6.8 min), pivalic acid (internal standard; 7.8 min), 2-methylbutyrate (11 min), isovalerate (12.9 min), and valerate (16.1 min). To prevent carryover effects between samples and to maintain similar conditions, between each sample distilled H2O was injected.

Estimation of VFA and Fluid Dynamics

Polyethylene glycol concentrations of samples taken at 0900 through 2400 h on the last 04 days of each period were composited across days and concentrations of samples at all time points 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. Rumen fluid volume was estimated by dividing the PEG bolus dose by the curve’s y-intercept. The rumen fluid volume was used to calculate VFA pool sizes by multiplying by VFA concentrations.

The pool sizes of VFA estimated across the time-series of samples collected were used to derive a 1 pool model of kinetics for each VFA. Entry into the pool was estimated as an exponential decay over time, to reflect the gradual depletion of substrate introduced into the rumen through feeding. The intercept of this exponential function was used to estimate the rate of maximum net apparent appearance (mmol/h), and the slope of the exponential function reflected the fractional rate of net apparent appearance decline (mmol/mmol/h). When solved, the exponential function reflected the apparent net appearance of the VFA for each point in time. This function was then solved across all time points and averaged, to yield the mean apparent net appearance (mmol/h), or summed, to yield the total apparent net appearance (mmol/12 h).

Exit from the VFA pool was estimated to occur through two routes: fluid mediated and net apparent disappearance. The fluid mediated passage was calculated as the product of the VFA in the pool at each time point (mmol) and the fluid mediated passage rate (%/h). The net apparent disappearance was calculated assuming mass action kinetics, and the rate constant reflected the rate of apparent disappearance (mmol/mmol/h). This rate of apparent disappearance was used, along with the VFA pool size at each time point to estimate the total net apparent disappearance (mmol/12 h), and the mean net apparent disappearance (mmol/h). The model parameters, which included the intercept and slope of the net apparent appearance equation and the slope of the net apparent disappearance equation, were derived using the FME (Soetaert, 2010) and deSolve packages (Soetaert et al., 2015) of R leveraging a 4th order Runge Kutta integration. After estimating the parameter values and calculating the associated fluxes for each animal in each period were analyzed through traditional statistical analysis for comparison of conclusions which might be drawn from the kinetics data compared with those drawn from the concentration or molar proportion data alone. Statistical Analysis

Statistical analyses were conducted in R version 4.1.2 (R Core Team, 2021) using the lme4 package (Bates et al., 2009) and lmerTest package (Kuznetsova et al., 2015). Response variables included fluid volume (L); fluid passage rate (%/h); fluid pH; VFA concentrations (mM); VFA molar proportions (% mol); VFA maximal rate of net appearance (mmol/h), the fitted decline in net apparent apperance rate (mmol/mmol/h), rate of absorption (mmol/mmol/h), net appearance (mmol/h) and net disappearance (mmol/h) of individual VFA. Response variables were analyzed using the following mixed effect 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. Analysis of variance (ANOVA) was performed for each variable using anova package (Ferreira et al., 2014) and estimated marginal means using emmeans package (Lenth et al., 2019) were 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 values for DMI (kg/d), rumen pH, rumen fluid volume (L), and fluid passage rate (%/h) are presented in Table 5-2. Dry matter intake and rumen pH were unaffected by protein source (P = 0.297 and 0.481 respectively) and fiber source (P = 0.632 and 0.201 respectively). Non-significant effects of protein or fiber source on DMI suggests that all the diets had a similar palatability and activation of feed intake regulatory mechanisms despite the differences in feed sources. Generally, fiber with long particles (i.e., TH) is expected to result in a slower passage rate in comparison with low particle size fiber (BP), and previous studies reported particle size differences affecting DMI (Bowman et al., 1991). However, the very different types of fiber presented by the TH and BP sources do not appear to result in intake shifts analogous to those resulting from altered grass maturity or legume substitution, supporting the idea that both particle size and chemical composition of feed influence DMI (White et al., 2017). In line with present results, (Mynhardt et al., 2006) observed no significant difference in DMI in Dohne Merino lambs fed with diets containing unprocessed and heat treated soybean as we used in this study. Several other studies in which diets consisted of different sources of protein including soybean meal vs coated urea (low rumen degradability) (Ravi Kanth Reddy et al., 2019), and soybean meal vs legume tree foliage (Best et al., 2017) also reported no significant differences in DMI of sheep. The consistent feed intakes irrespective of fiber or protein source are also consistent among studies, as previous work done with these same ingredients also found no influence of diet on DMI of sheep (Gleason et al., 2022a). The consistent DMI in this study is helpful as a means to explore how VFA supplies, and efficiency of production differ from diets with similar intakes.

Wethers in this study were consuming a high concentrate diet, and accordingly rumen pH ranged between 5.98 to 6.27 (Table 5-2) which was slightly lower than the standard normal pH range of 6.4 to 6.8 (Jasmin et al., 2011), and unaffected by diet. Ruminal pH ranges lower than 5.8 are defined as sub-acute rumen acidosis (SARA) which is a result of high percentage of highly fermentable carbohydrate and low percentage of physically effective fiber content common in concentrate based diets (Kleen et al., 2003). Although BP contributes to low level of physically effective fiber (Teimouri Yansari, 2017), and its fiber fraction consists largely of neutral detergent soluble fiber (NDSF), mainly pectin (Yapo et al., 2007), and numerous studies have reported pH stabilizing effect of BP (Mojtahedi and Mesgaran, 2011; Shahmoradi et al., 2016). Regardless of rapid degradability of pectin in the rumen (Marounek et al., 1985), pectin fermentation produces less lactate (Jaramillo-López et al., 2017; Münnich et al., 2017) in comparison to most of the other readily fermentable carbohydrate sources. Therefore, BP based diets are often reported to maintain the normal pH range in the rumen (Münnich et al., 2017; Ali et al., 2019), as was observed in the present study. Timothy-hay-based diets were also expected to maintain rumen pH in the normal range, as TH supplied adequate physically effective fiber, and the similar pH observed in these diets supports these hypothesized fermentation characteristics. The similarity in pH among diets also supports the assertion that thermodynamic conditions were likely somewhat similar on these diets, and therefore interconversion of VFA may be more consistent than if thermodynamic conditions varied considerably.

Fluid volume and passage rate were affected by protein source (P = 0.033), and HSBM resulted in greater fluid volume and lower passage rates. The average pool sizes we observed are in line with the values reported by much earlier studies by Bergman et al., (1965) and (Ulyatt, 1964) where rumen volume ranged approximately between 3 to 7 L in sheep fed with forage-based diets, but slightly lower than 6.7 and 7.15 L in more recent work (Nolan et al., 2010). Although no significant differences in rumen volume were observed in a study by (Gleason et al., 2022a), where they used the same diets and animals as we used in the current study, we have greater confidence in the fluid data obtained from the present work due to better physiological coherence. Fluid passage rate plays an important role in rumen fermentation because it influences digestion, absorption and availability of nutrients, and the amount of VFA that exit the rumen with fluid (López et al., 2003). The higher supply of rumen degradable protein (RDP) in SBM diets may have reduced the rumen retention time thus increasing the fluid passage rate on SBM vs HSBM diets. The fractional rate of passage we estimated in our study were similar to Moyo et al., (Moyo et al., 2017) where they estimated 7.4 %/h using an artificial neural network, and in agreement with Seo et al., (Seo et al., 2006) who reported estimations of 4.16 %/h for forage based diets, and 6.27 %/h for concentrate based diets.

Rumen fluid dynamics are critical measurements for investigations of dynamic processes such as appearance and disappearance of VFA in the rumen. We used PEG as a fluid marker for several reasons. First, PEG was expected to associate exclusively with the liquid phase of rumen digesta (Warner and Stacy, 1968). PEG is thought to be more stable in comparison to EDTA complexes commonly used, which are either challenging to purchase or dissociate in conditions similar to those encountered in the rumen (Hall and Van Soest, 2019). Finally, considering the interest in a rapid and inexpensive screening protocol for VFA, PEG is particularly attractive due to its cost effectiveness, ease of preparation, and scalability of analysis (Clemens, 1982). However, this work and our previous studies (Gleason et al., 2022a) using PEG as a fluid marker based on bolus administration show great variability in outcomes, even within the same animal on different days, suggesting that more robust measurement protocols (such as bailing the rumen and leveraging a continuous infusion) are preferable for future studies reliant on fluid dynamics estimates.

Volatile Fatty Acid Dynamics

Hall et al (2015) noted that VFA concentrations are in incomplete means of exploring fermentation profiles and VFA supplies, due to the confounding influence of rumen fluid volume and passage rate. Molar proportions were advocated as better representations of the profile of VFA produced, though still incomplete metrics of VFA supplies for animal use. Indeed, in this work, when comparing the conclusions drawn from concentration and molar proportion data to those drawn from the VFA kinetics assessment, there are notable differences. Although we are unable to make comparisons among the kinetics assessment to gold-standard measures of VFA fluxes (i.e., isotope-based studies) due to the differences in rumen dynamics driven by meal-feeding versus artificially induced metabolic-steady-state, we can use these kinetics data as a represent of the rates and extent of transfer of VFA (to various metabolic or microbial endpoints) under differing dietary conditions that produce similar feed nutrient intake and similar pH. Indeed, the confounding influence of differing fluid dynamics and expected differences in rumen degradability of protein and fiber, led to considerable shifts in interpretation related to fermentation outcomes when comparing the concentration and molar proportion data in the context of the added details from the kinetics assessment.

Fiber source significantly affected the concentration of acetate and butyrate, and the molar proportions of valerate, isovalerate, and iso-butyrate (Table 5-3); however, the kinetics assessment yielded numerous effects of the interaction of protein and fiber, as well as several independent effects of protein source (Table 5-4). Although the concentration and molar proportion data are able to inform researchers about the average conditions in the rumen throughout the fermentation, the kinetics data can help explain how those averages come about. This more precise understanding about the rate of fermentation and the quantities of VFA in flux throughout fermentation are an important addition which shifts physiological understanding of the concentration and molar proportion data.

Acetate concentrations were elevated on BP diets compared with TH (Table 5-3), which was in agreement with previous studies (Zhao et al., 2013; Asadollahi et al., 2016; Gleason et al., 2022a); however, the maximal appearance rate of acetate, the rate of appearance decline, and the disappearance rate were influenced by the interaction of protein and fiber source. Diets using BP had moderate maximal appearance rates compared with TH diets, and lower rates of decline in appearance rates, suggesting a slower and more moderate appearance of acetate over the course of fermentation. This is in contrast to the TH-HSBM diet, which yielded high maximal appearance and high appearance decline suggesting a rapid initial appearance of acetate, which dissipated quickly, and the TH-SBM diet which resulted in low maximal appearance and fast appearance decline. Although the pattern of acetate appearance differed considerably among these groups, which supported differences in the average acetate concentration, there was no influence of diet on the mean appearance rates among the diets. Disappearance of acetate was slowest on the BP-HSBM diet, and fastest on the TH-HSBM diet, and the differences in kinetics and concentrations supported the highest disappearance flux on the TH-HSBM diet, followed by the BP-HSBM and BP-SBM diets, with the lowest flux associated with the TH-SBM diet. If analogous to availability of acetate to microbial and metabolic endpoints, the data from the acetate disappearance estimate suggests a very different conclusion than the acetate concentrations. TH-HSBM had the lowest acetate concentration, likely due to having a more rapid, and greater mass of, acetate disappearance.

Similar dynamics were identified for butyrate, which had concentrations significantly influenced by fiber source, but kinetics driven by the interaction of fiber and protein sources. Pectin is a primary substrate for butyrate-producing *P. brevis* activity and protozoal activity (Nograšek et al., 2015), and has been thought to drive enhanced concentrations and molar proportions of butyrate associated with BP diets in this and previous (Abo-Zeid et al., 2017; Guo et al., 2021) studies. Despite these understood differences, the kinetics data suggest alternative explanations. The TH-HSBM diets resulted in the greatest maximal appearance, the fastest rate of disappearance, the greatest appearance flux, the fastest rate of disappearance, and the greatest disappearance flux. The BP rations resulted in intermediate butyrate appearance, and disappearance fluxes, while the TH-SBM ration yielded the lowest fluxes. Again, although the TH diets would be expected to have the lowest butyrate availability based on concentration differences, the kinetics assessment suggests the low concentration on the TH-HSBM was driven by rapid rates of appearance and disappearance, with greater disappearance flux. In contrast, the low TH-SBM concentrations were driven by low appearance.

The acetate and butyrate data collectively suggest a unique fermentation profile on the TH-HSBM diets that supported elevated availability of acetate and butyrate for microbial and metabolic functions. In an in-situ exploration of BP compared with TH in heifers, Beckett et al., (2021) identified that BP diets yielded greater ruminal fiber disappearance than did TH diets. These fiber degradation dynamics on BP diets is somewhat contrary to the elevated VFA fluxes identified in the present work. However, Hall et al., (2010) found elevated in situ NDF digestibility at 30 h for heat treated soybean meal compared with regular soybean meal, and more moderate rumen pH, perhaps suggesting different fermentation kinetics and elevated fiber breakdown associated with HSBM compared with SBM. In sheep, TH diets were associated with increased bacterial diversity (Gleason et al., 2021), and TH-HSBM diets tended to support elevated monocarboxylate 2 transporter gene expression (Gleason et al., 2022a).

In a different example, neither propionate concentrations nor molar proportions were influenced by diet (Table 5-3); however, the rate of appearance decline, and the disappearance rate of propionate were influenced by the interaction of protein and fiber source. These rate estimates also supported faster appearance rate decline and faster disappearance on the TH-HSBM diet compared with other diets. Although mean appearance was not influenced by diet, mean disappearance was affected by protein source, with the HSBM diets resulting in greater mean disappearance. Although the concentration data would support equal propionate availability on all diets, the kinetics assessment indicated similar synthesis but differences in disappearance driven by protein source.

The molar proportions of valerate, isovalerate, and iso-butyrate were all influenced by fiber source, with TH yielding higher concentrations than BP. The kinetics exploration of these VFA suggested protein source influenced appearance of isovalerate, and disappearance of isovalerate and isobutyrate. In both cases, the HSBM protein source resulted in greater flux of VFA than did the SBM. Production of iso-acid is thought to be largely driven by the availability and rate of degradation of feed proteins in the rumen (Ropotă et al., 2016). In an in vitro study in which 3 protein sources were evaluated for their capacity to conversion of branched chain amino acids to corresponding isoacids (Apajalahti et al., 2019) higher RUP sources resulted in lower isoacid concentration which is in agreement with our observations. Most of the ruminal fibrolytic microbes require iso-acids to synthesize branched-chain amino acids for proliferation and improve their functions and enzymatic activities (Andries et al., 1987), therefore the elevated appearance of isoacid on the HSBM diets may partially explain the greater fermentation outcomes on the TH-HSBM diets among major VFA as well. These same dynamics were not supported when concentrations and molar proportions were viewed along, providing support to the idea that kinetic assessment of fermentation outcomes yields opportunity for more mechanistic understanding.

Limitations

One major limitation of the present work is the inability to characterize ruminal VFA interconversions. Disappearance, although ideally representative of VFA available to the host animals, in practice represents VFA availability for both host and microbial processes. These microbial processes can reflect interconversion among VFA, such as the bidirectional transfer of acetate to butyrate and back. It can also reflect sequential metabolic pathways, such as those linking butyrate and valerate (Kristensen et al., 2000; Kristensen and Harmon, 2004) , or highlighting valerate as a condensation of acetate and propionate (Wiltrout and Satter, 1972). In the case of butyrate and acetate, most of the dynamics observed in the kinetics results were similar between these VFA, meaning that the same diets were identified to have faster rates or greater fluxes. If interconversion were driving major supply differences among these VFA, we would expect to see some diets where the equilibrium favored acetate, and others where it favored butyrate. However, the consistencies observed here may have been supported by the consistent rumen pH. To better contextualize the opportunities to interpret these data when VFA interconversions are expected to be different, diets differing dramatically in thermodynamic conditions should be explored.

Another limitation of the present work is the reliance on fluid marker dynamics. In our previous studies, fluid markers have proven variable and, in some cases unreliable, estimates of dynamics. In this work, we leveraged 4 sequential days of fluid marker measurement to better contextualize the variability in data. This repeated assessment of fluid volume and passage rate was able to produce more physiologically coherent estimates of fluid dynamics than observed in previous studies (Gleason et al., 2022a); however, the variation around these numbers, and the sensitivity of the procedure to accurate fluid dynamics measurements necessitates further exploration into more reliable, consistent, and continuous exploration of fluid dynamics within the rumen. This is particularly the case in the meal-feeding scenario tested herein, because fluid dynamics are not expected to remain constant over the course of fermentation. Thus, although the PEG data provide an overall average estimate, they fail to support the details needed to characterize the real-time variability in rumen fluid volume. Understanding this variability is essential to better estimating more representative time-series fermentation dynamics.

CONCLUSION

The study explored a lower-cost and faster screening method for evaluating the dynamics of rumen fermentation as an alternative to expensive isotope based assessments. However, the estimates of apparent rates derived from this approach cannot be directly interpreted as masses of energy substrate provided to the animal, due to lack of capacity to explore the complicating factor of interconversions among VFA. More work needs to refine the current approach with greater emphasis on characterizing kinetics across a variety of thermodynamic states, and using fluid volume monitoring that supports real-time, consistent, and reliable measurement. Pending further exploration of these limitations, this simple approach to estimate appearance and disappearance of individual VFA using a one pool model may pave the way for feasible investigations of VFA dynamics in the rumen, and eventually, supplies to the animal.

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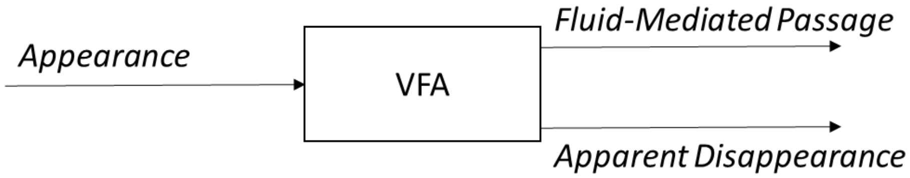
Figures

Figure 5-1. Illustration of one pool model.



$$dVFA_t = Appearance_t - FluidMediatedPassage_t - ApparentDisappearance_t$$

$$Appearance_t = a \times e^{-bt}$$

$$FluidMediatedPassage_t = FluidPassage \times [VFA_t]$$

$$ApparentDisappearance_t = c \times VFA_t$$

The entry to the pool was estimated as an exponential function of time relative to feeding, and the exit rate was assumed to be mass-action.

Tables

Table 5-1. Ingredients and nutrient composition for each treatment diet.

	BP-HSBM	TH-HSBM	BP-SBM	TH-SBM
Ingredient, % DM				
Alfalfa hay	10.1	9.9	10.1	10.0
Corn	19.9	15.7	10.1	16.0
Barley	19.9	15.7	19.2	16.0
Soybean meal	0.00	0.00	16.1	13.9
Soyplus	18.1	14.4	0.00	0.00
Timothy hay	0.00	20.6	0.00	23.5
Beet pulp, pelleted	41.0	0.00	42.0	0.00
Wheat middlings	0.50	23.0	2.20	20.0
Trace mineral salt	0.55	0.88	0.54	0.74
Vitamin premix	0.55	0.88	0.54	0.74
Nutrient, %				
DM	89.49	88.50	89.16	88.67
CP	12.72	12.64	12.99	13.00
NDF	24.75	24.62	24.22	24.97
ADF	16.31	17.11	16.15	17.81
Lignin	2.27	2.39	2.30	2.33
Fat	2.40	3.00	1.63	2.53
OM	93.06	93.38	92.79	93.58
Ash	6.94	6.62	7.21	6.42

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

Table 5-2. 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.

Item	Diet				SEM	P value		
	BP-HSBM	TH-HSBM	BP-SBM	TH-SBM		Protein	Fiber	Protein× Fiber
DMI, kg	3.92	3.90	3.70	3.84	0.288	0.297	0.632	0.559
Rumen pH	6.22	6.21	6.27	5.98	0.183	0.481	0.201	0.336
Rumen fluid volume, L	5.60	6.63	4.88	4.74	0.651	0.033	0.435	0.313
Rumen fluid Passage rate, %/h	4.60	3.26	5.55	6.60	1.212	0.048	0.889	0.270

BP-HSBM: beet pulp and heat treated soybean meal, TH-HSBM: timothy hay and heat treated soybean meal, BP-SBM: beet pulp and soybean meal, TH-SBM: timothy hay soybean meal.

Table 5-3. 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.

Item	Diet				SEM	P value		
	BP-HSBM	TH-HSBM	BP-SBM	TH-SBM		Protein	Fiber	Protein×Fiber
Concentration, mM								
Acetate	67.5	50.7	70.1	57.2	4.57	0.272	0.002	0.623
Propionate	28.7	26.7	28.2	29.8	4.59	0.723	0.966	0.653
Butyrate	13.89	10.89	12.83	9.99	1.02	0.296	0.004	0.930
Valerate	4.17	3.94	3.81	5.07	0.73	0.522	0.381	0.228
Isovalerate	0.472	0.432	0.578	0.539	0.119	0.197	0.602	0.992
Iso-butyrate	0.630	0.594	0.770	0.749	0.132	0.128	0.746	0.945
Molar proportion, % mol								
Acetate	58.7	55.5	60.0	53.8	3.02	0.920	0.081	0.580
Propionate	23.8	26.5	23.5	29.2	3.09	0.649	0.120	0.568
Butyrate	12.07	11.71	11.03	9.51	0.980	0.071	0.277	0.501
Valerate	3.54	4.08	3.15	4.76	0.646	0.771	0.035	0.286
Isovalerate	0.458	0.599	0.498	0.639	0.100	0.544	0.031	0.996
Iso-butyrate	0.607	0.815	0.677	0.860	0.110	0.468	0.016	0.882

BP-HSBM: beet pulp and heat treated soybean meal, TH-HSBM: timothy hay and heat treated soybean meal, BP-SBM: beet pulp and soybean meal, TH-SBM: timothy hay soybean meal.

Table 5-4. Appearance and disappearance rates of individual VFA

Item	Diet				SEM	P value		
	BP-HSBM	TH-HSBM	BP-SBM	TH-SBM		Protein	Fiber	Protein× Fiber
Maximum Appearance Rate (mmol/h)								
Acetate	92.4a	135.5b	104ab	90.6a	10.67	0.066	0.089	0.006
Propionate	43.8	68	36.2	42.7	13.12	0.112	0.100	0.417
Butyrate	20a	38.7b	24a	19.9a	4.12	0.040	0.039	0.005
Valerate	8.49	13.13	7.61	10.16	2.00	0.242	0.036	0.538
Isovalerate	1.97	1.55	1.22	0.372	0.573	0.032	0.129	0.658
Isobutyrate	1.96	2.39	1.19	1.28	0.830	0.100	0.621	0.789
Rate of Appearance Decline (mmol/mmol/h)								
Acetate	0.098a	0.148b	0.109ab	0.112ab	0.012	0.214	0.016	0.029
Propionate	0.106	0.124	0.096	0.105	0.016	0.004	0.007	0.425
Butyrate	0.106	0.181	0.126	0.142	0.027	0.707	0.065	0.231
Valerate	0.125	0.161	0.136	0.132	0.023	0.614	0.389	0.300
Isovalerate	0.10	0.10	0.15	0.15	0.039	0.175	0.999	0.999
Isobutyrate	0.087	0.169	0.153	0.154	0.050	0.498	0.255	0.341
Mean Appearance (mmol/h)								
Acetate	51.2	60.6	55.8	45.8	5.3	0.276	0.947	0.056
Propionate	22.9	31	21.1	23	5.55	0.254	0.230	0.512
Butyrate	10.02	15.6	12.18	8.31	1.732	0.114	0.586	0.006
Valerate	4.05	5.32	3.79	4.60	0.877	0.464	0.120	0.740
Isovalerate	1.09	0.855	0.667	0.200	0.318	0.030	0.131	0.666
Isobutyrate	1.08	1.31	0.651	0.705	0.461	0.100	0.638	0.813
Disappearance Rate (mmol/mmol/h)								
Acetate	0.072a	0.100b	0.075ab	0.076ab	0.007	0.098	0.019	0.044
Propionate	0.071	0.088	0.069	0.071	0.003	0.001	<0.001	0.009
Butyrate	0.075	0.132	0.103	0.074	0.018	0.359	0.415	0.017
Valerate	0.0934	0.131	0.105	0.076	0.026	0.361	0.859	0.169

Isovalerate	0.538	0.470	0.373	0.256	0.175	0.183	0.489	0.868
Isobutyrate	0.440	0.503	0.155	0.361	0.165	0.106	0.284	0.613
Mean Disappearance (mmol/h)								
Acetate	26.2	34.1	25.8	20.6	3.95	0.031	0.627	0.055
Propionate	11.09	17.28	9.38	9.11	2.88	0.033	0.170	0.191
Butyrate	5.93	9.43	6.31	3.62	1.38	0.037	0.738	0.024
Valerate	1.93	2.96	1.92	1.91	0.521	0.221	0.225	0.234
Isovalerate	1.5a	1.378	0.803	0.263b	0.449	0.009	0.271	0.551
Isobutyrate	1.50	1.95	0.721	0.782	0.590	0.017	0.471	0.644

BP-HSBM: beet pulp and heat treated soybean meal, TH-HSBM: timothy hay and heat treated soybean meal, BP-SBM: beet pulp and soybean meal, TH-SBM: timothy hay soybean meal.

Chapter 6 : Finisher Lamb Growth and Rumen Fermentation Responses to the Plane of Nutrition and Naturally Occurring Coccidiosis

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ABSTRACT

The objective of the present study was to investigate the interaction of plane of nutrition and naturally occurring coccidiosis on finisher lamb growth performance, FAMACHA score, and rumen volatile fatty acid profile. The study included 30 Suffolk, Dorset or Suffolk x Dorset lambs and were divided into 2 groups based on their initial body weight and assigned to 2 feeding groups differing in dietary energy intake to create lambs representing divergent growth curves due to differing nutritional management. Lambs with naturally occurring coccidiosis and healthy lambs were present in both feeding groups making a 2x2 factorial arrangement of treatments, a) high plane of nutrition (HPN) lambs with no clinical coccidiosis diagnosis (HPNH), b) HPN lambs with clinical coccidiosis (HPNC), c) low plane of nutrition (LPN) lambs with no clinical coccidiosis diagnosis (LPNH), d) LPN lambs with clinical coccidiosis (LPNC). Body weight and FAMACHA scores were recorded once every 2 weeks. On d 65 of feeding, lambs were slaughtered, and rumen fluid samples were collected and analyzed for volatile fatty acid concentrations. All response variables were analyzed statistically using a linear mixed effects model with fixed effects for plane of nutrition, health status, and a random effect for initial body weight nested within the pen. The total and average weight gain were not associated with planes of nutrition, health status, or the interaction. Health status had an impact on FAMACHA© score ($P = 0.047$) and concentration of isobutyrate ($P = 0.037$) and tended to affect total VFA ($P = 0.085$) and acetate ($P = 0.071$) concentrations. The interaction between the plane of nutrition and the health status tended to affect butyrate concentration ($P = 0.058$). These data support the conclusion that coccidiosis infection impacted on rumen fermentation in a manner independent of the plane of nutrition; however, the translation of these rumen level impacts did not translate to the production responses.

Keywords: Anemia**,** average daily gain, *Eimeria* spp., FAMACHA©, volatile fatty acid

INTRODUCTION

Per capita lamb consumption in the U.S. is at a 20-year high, reaching 0.62 kg in 2021 (Association, 2021). Despite high consumption, the U.S. sheep inventory is at all-time low of approximately 5.2 million sheep and lambs in 2019 (NAHMS, 2021). This discrepancy is largely because the domestic sheep industry fulfills only 50% of the U.S. demand for lamb meat and less than 30% of the demand for wool (Purcell and Tech, 2007). Failure to meet this demand has resulted in increased levels of sheep meat and wool importation in the U.S. (Association, 2021). Based on these market shifts, and the fact that the sheep industry is vital to the livelihood of many farmers throughout the country (Ruminants et al., 2007), there is potential for expanding the commercial sheep industry in the U.S. Additionally, sheep are used as a natural, low-cost means of managing rangelands, forests, and agricultural lands, because most of the nutrient requirements of mature sheep can be achieved through grazing, making sheep production suitable for low-input production environments.

Meeting adequate nutrition requirements of young lambs with high potential for live weight gain (Ruminants et al., 2007), however, requires more intensive feeding strategies. According to the NRC (Ruminants et al., 2007), young lambs, between 60 and 150 days of age, need diets with high protein and energy content which exceed the levels supplied in most forages used for sheep rearing. With seasonal fluctuations of forage quality most of the hay used for sheep feeding is low in crude protein and essential macro and micro minerals (Griggs et al., 2008; McCartney et al., 2009). Therefore, to obtain high growth performance, diets with a high proportion of concentrate are typically used for finishing lambs. A great deal of research shows that lambs grow faster on concentrate-based diets than on forage-based diets (Fimbres et al., 2002a; Demirel et al., 2006; Archimède et al., 2008). However, these feeding strategies often involve confinement feeding of lambs, resulting in challenges associated with maintaining flock health (NAHMS, 2021).

Maintaining a healthy flock is imperative for successful sheep operations because disease compromises overall growth rate, immunity, and reproductive performance, all leading to substantial economic losses (Odden et al., 2017; Dudko et al., 2018). The Animal and Plant Health Inspection Service of U.S. Department of Agriculture reported that internal parasites caused approximately 16 % loss in lambs (NAHMS, 2021) and a survey conducted by the same authority reported that gastrointestinal (GI) parasites are the number one health concern among sheep stakeholders (NAHMS, 2021). A 7-year review of clinical cases at Auburn University Veterinary Medical Teaching Hospital in Alabama found that parasite infection was the primary reason that 70% of sheep and 91% of goats were examined and treated by hospital clinicians (Pugh and Navarre, 2001). Gastrointestinal infestation with parasites is a particularly challenging situation because the infestation both increases cost of energy requirements due to activation of immune responses, and -depresses rumen function and negative consequences on metabolism (Mavrot et al., 2015) leading to lower energy supply to the animal (Jacobson et al., 2020). Coccidiosis is a major parasitic infection caused by *Eimeria* spp. protozoan that commonly infects the small and large intestines of sheep (Raue et al., 2017). Symptoms of clinical coccidiosis are diarrhea, dehydration, decreased appetite, weight loss, and death (Chartier and Paraud, 2012; Andrews, 2013). The effects of *Eimeria* infections on animals are thought to depend on the environmental situation, immunity of the animals (Gregory et al., 1983), and the plane of nutrition. Studies focused on controlling coccidiosis are abundant in literature (Fitzgerald and Mansfield, 1978; Taylor et al., 2003; Saratsis et al., 2016; de Souza Rodrigues et al., 2020) but these studies are limited in exploring the interaction between plane of nutrition and naturally occurring coccidia on growth performance and rumen fermentation. Most of the coccidia associated studies have focused on the lower GI tract since it is the primary site of pathological change. Nevertheless, impairment in the gastrointestinal tract may affect the function and activity of the rumen, contributing to impaired volatile fatty acid (VFA) production and reduced energy supplies to the animal. Because VFA are the main energy source for ruminants (Bergman, 1990) and considering the cost of energy to maintain immunity and nutrient metabolism when lambs are under infection, it is worthwhile to investigate the effect of plane of nutrition and coccidiosis on rumen VFA production. Thus, the objective of this study was to explore how dietary manipulation and naturally occurring disease interact to influence animal performance and fermentation outcomes which will help to implement better health and nutritional management protocols.

MATERIALS AND METHODS

Animals, Diets and Experimental Design

All the procedures and animal use described in this study were approved by the Virginia Tech Institutional Animal Care and Use Committee (Protocol #20-175). The study included 30 commercial wethers (Suffolk, Dorset or Suffolk x Dorset) that were group fed in a standard production feedlot (330 m^2) at the Smithfield Farm, Virginia Tech, Blacksburg, VA. Prior to initiating the experiment all lambs were dewormed using fenbendazole (Panacur 10 mg/kg body weight, Merck Animal Heath, DE, USA) and Levamisole Hydrochloride (Prohibit at 8 mg/kg of body weight, AgriLabs, MO, USA), per standard industry management and veterinary recommendation. Deworming occurred 3 weeks prior to the start of the experiment and was not expected to interfere with the experimental measurements. Lambs were between 04 - 06 months of age at the start of the study. The lambs were classified by initial body weight targeting different finishing weights to cater market demands: low (28.4 ± 4.31 kg) and high (36.1 ±2.37 kg). Lambs were fed either 1) a low plane of nutrition (LPN) diet targeting 100 g/d weight gain or, 2) a high plane of nutrition (HPN) diet targeting 200 g/d weight gain. Due to market preferences, there is considerable variation in production systems regarding rates of lamb growth in the U.S (Thorne et al., 2021). Including two planes of nutrition in this study allowed for generalization of results among different growth trajectories of finishing lambs.

Grouping animals based on starting weights was reflective of industry settings where animals are grouped and purchased based on body weight. Body weight at the start of the study would be an emergent property of health, nutritional, genetic, and environmental factors, and is a complex phenotype that should not be conflated to reflect only differences in planes of nutrition.

Across plane of nutrition groups, lambs averaged 32.1 ± 4.5 kg body weight at the beginning of the experiment. After introduction to the feedlot environment, animals were naturally infected with Eimeria spp. Animals began showing signs of coccidiosis after 13 days, and both groups of animals received herd-level coccidiosis treatment with amprolium (Corid 9.6 % solution, 8 mg of amprolium per 1kg of body weight for 5 consecutive days). Animals did not have a history of anticoccidial treatment prior to entering the feedlot.

Out of 30 lambs, 04 lambs died (1 due to coccidiosis, 2 due to pneumonia and coccidiosis, and 1 for undiagnosed reasons) during the experimental period and those lambs were excluded from the data set. Of the 26 lambs that completed the trial, 9 lambs were diagnosed with clinical coccidiosis and recovered after treatment (infected lambs were individually treated following the same protocol as herd level treatment). The low nutritional group consisted of 14 lambs of which 6 lambs were diagnosed with coccidiosis. The HPN group consisted of 12 lambs, of which 3 lambs were diagnosed with coccidiosis. The resulting 4 treatment groups were a) HPN lambs with no clinical coccidiosis diagnosis (HPNH), b) HPN lambs with clinical coccidiosis (HPNC), c) LPN lambs with no clinical coccidiosis diagnosis (LPNH), and d) LPN lambs with clinical coccidiosis (LPNC). Diets in both groups were consisted with ad libitum grass hay and a commercial concentrate (Cargill Animal Nutrition, Minneapolis, MN, USA) supplement and were fed twice daily at 0900 hr and 1700 hr. Lambs in the LPN group received 0.45 kg of concentrate per lamb and 0.90 kg of concentrate per lamb in the HPN group. Target was to provide two levels of energy to achieve two different ADG. Both groups had access to fresh, clean water and mineral supplements throughout the day.

Measurements, Sampling and Laboratory Analysis

Body weight and FAMACHA© scores were recorded once every 2 weeks. The FAMACHA system was used to determine the lower eye mucous membrane color that correspond to different levels of anemia: 1 = red, non-anemic; 2 = red-pink, non-anemic; 3 = pink, mildly anemic; 4 = pink-white, anemic; 5 = white, severely anemic (Van Wyk and Bath, 2002). Although the FAMACHA© is developed to assess anemia caused by *Haemonchus* species, the tool has been used as an anemia indicator caused by a wide range of gastrointestinal parasites including *Eimeria* species (Nurzaty Ewani et al., 2014; Meradi and Bentounsi, 2021). Previous studies by Nurzaty Ewani et al., (Nurzaty Ewani et al., 2014) and Wang et al., (Wang et al., 2010) observed anemia and diarrhea associated with lambs infected with *Eimeria* spp. Further, if lambs have more *Haemonchus*, this likely suppresses immune system and lambs will more likely be clinical for coccidia. Lambs were slaughtered on d 65 of the experiment and rumen fluid samples were collected from each animal promptly after slaughter. Samples were collected and processed in the laboratory and stored in glass vials at -20 ˚C until further analysis. Approximately 100 g of concentrate and grass hay were collected weekly, composited, and stored in -20°C until analysis.

Volatile fatty acid concentrations were analyzed using gas chromatography. Concentration of total VFA and individual VFA per lamb were determined using a Hewlett-Packard 5890 series gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a glass packed column (23110-U, Sigma-Aldrich) with N_2 as a carrier gas (24 mL/min). The inlet was 150°C, the flame ionization detector was 180°C, and the oven temperature was 175°C. Each run was 18 min long to separate acetate (retention time: 1.6 min), propionate (3.0 min), isobutyrate (5.2 min), butyrate (6.8 min), pivalic acid (internal standard; 7.8 min), 2-

methylbutyrate (11 min), isovalerate (12.9 min), and valerate (16.1 min). To prevent carryover effects between samples and to maintain similar conditions, between each sample distilled H_2O was injected. 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). Proximate analysis of feed samples was done by an external laboratory (Cumberland Valley Analytical Services, Waynesboro, PA). Analyses included DM (Goering and Van Soest, 1970; Shreve et al., 2006), N (method 990.03; Leco FP-528 Nitrogen Combustion Analyzer, Leco Corp., St. Joseph, MI), ADF [method 973.18; (Horwitz and Latimer, 2000)], NDF (Van Soest et al., 1991), lignin (Goering and Van Soest, 1970), starch (Hall, 2009), ash [method 942.05; (Horwitz and Latimer, 2000)], and minerals by inductively coupled plasma [method 985.01; (Horwitz and Latimer, 2000)].

Statistical Analysis

Statistical analyses were conducted in R version 4.1.2 (Team, 2021) using the lme4 and lmerTest packages (Bates et al., 2014). Response variables included were total body weight gain, average daily gain, FAMACHA score and concentrations and molar proportions of individual VFA. The following model was fitted for all variables:

$$Yijklm = \mu + Ni + Hj + (NHij) + BWk(l) + Pl + eijklm,$$

where Yijklm is the dependent variable, μ is the overall mean, Ni is the fixed effect of the plane of nutrition, Hj is the fixed effect of health status, NHij is the interaction effect, BWk is the random effect of initial body weight of individual lamb nested within the pen, and eijkl is the residual error. Analysis of variance (ANOVA) was performed for each variable and estimated marginal means were calculated using the emmeans package (Lenth et al., 2019). Significance was declared at $P < 0.05$ and a tendency considered when $0.05 \leq P < 0.10$. Importantly, in our statistical analysis, we used the effect of initial body weight to explore how group responses might differ due to initial differences in the animals (expressed as differences in body weight) versus those differences based on the nutritional treatments. Due to a failure in sampling of rumen fluid, data from 2 animals from HPNH group were not used for the statistical analysis.

RESULTS AND DISCUSSION

Growth Performance and FAMACHA Score

The effects of the plane of nutrition and health status on growth performance and FAMACHA© score of lambs are presented in Table 6-2. The plane of nutrition, status of health or the interaction did not have an effect on total gain or ADG. Numerically, the highest WG and ADG were associated with HPNH group, and values in other groups were not significantly different from each other. Although the numerical differences support the idea that nutrition and health interact to support performance, the variability around individual animal performance within the study precluded identification of statistically significant relationships. Status of health had an effect on FAMACHA© scores (P = 0.047) and lower values were associated with healthy groups irrespective of the plane of nutrition, with numerically higher FAMACHA© scores were associated with coccidiosis conditions.

Rumen Volatile Fatty Acid Profile

Concentrations and molar proportions of individual VFA are presented in Table 6-3. Health status caused by the coccidiosis infection had an effect on concentration of isobutyrate (P = 0.037) and tended to alter the concentrations of total VFA (P = 0.085) and acetate (P = 0.071). The interaction effect of the plane of nutrition and health status tended to affect butyrate concentrations (P = 0.058). Molar proportions of individual VFA were not affected by the plane of nutrition, health status or the interaction. Numerically, concentrations of total VFA and individual VFA in HPNH, HPNC and LPNH groups were quite similar to one another, while the LPNC group had the lowest reported values.

Growth Performance and FAMACHA© Score

According to the NRC (Ruminants et al., 2007) guidelines for sheep nutrient requirements, 4 to 7 month old finisher lambs should achieve an ADG of range of 205 to 295 g. In the present study, irrespective of the plane of nutrition and health status neither group reached the recommended ADG, partially because diets were formulated to target lower rates of gain than suggested in the NRC (Ruminants et al., 2007). Despite the highest ADG of 87.5 g associated with the HPNH group; it is still less than 50% of the target used in formulation. Possible explanations for the limited growth rates observed in the present study could be poor feed intake and forage quality. Based on the chemical analysis of the forage and concentrates (Table 6-1) fed to the lambs, we observed that grass hay was lower in protein supply of 7.7 % and higher NDF of 69 %. This forage was quite mature and likely had poor digestibility. Another possible explanation for lower than the standard ADG we observed in the HPNH group is the negative impact of whole herd coccidiosis treatment and subclinical coccidiosis. Coccidiosis treatment is known to depress feed and water intake, thus may have negatively impacted productivity of animals. Further, the effect of coccidiosis on weight gain and feed efficiency has been inconsistent in studies and it is even more challenging to ascertain the influence of subclinical infections (De la Fuente et al., 1993). Nevertheless, subclinical coccidiosis may contribute to low weight gain, reduced feed intake and feed utilization (Gauly et al., 2004; Kaya, 2004). However, the lower ADG observed in the present study closely follows the ADG values reported in several previous studies (Atti and Mahouachi, 2011; Bhatt and Sahoo, 2019). In Atti and Mahouachi (Atti and Mahouachi, 2011)they observed the highest ADG of 108g/day for the high nutrition group and 61 g/day for the low nutrition group while Bhatt and Sahoo (Bhatt and Sahoo, 2019) reported 99 to 140 g/day ADG values. In contrast to present results which did not show an effect of nutritional plane on total weight gain, numerous previous studies have linked plane of nutrition and growth performance. For example, a previous study (Hegarty et al., 2006) reported that the plane of nutrition had a dramatic effect on lamb live weight, with low and high lambs differing in weight by 9.1 kg (P<0.001) at weaning and by 14.9 kg at slaughter. Indeed, standard understanding of energetics also supports the expectation that lambs fed greater energy content diets should grow more rapidly. Although we observed numerically higher total weight gains and ADG in the HPN group compared with the LPN group, the variability induced by the added health challenge, and the small number of animals used in each group may help explain why these numerical differences did not approach statistical significance. Indeed, our numerical differences are sensible given the basic understanding of nutritional energetics and concur well with other previous work (Fimbres et al., 2002b; Papi et al., 2011) reporting positive gain responses in feedlot lambs with dietary concentrate.

A major limitation of implementing a selective treatment approach for parasitic infections has been the lack of an efficient and economical means of identifying those animals' requiring treatment. To address this issue, FAMACHA score has been utilized successfully in African countries (Ejlertsen et al., 2006; Sissay et al., 2007) and the United States (Kaplan et al., 2004; Burke et al., 2007). As shown in Table 6-2, FAMACHA© score did not differ between groups in response to the plane of nutrition but was affected by the health status (P = 0.047). The highest FAMACHA© score was observed in the LPNC group and is indicative of anemia due to the impaired ability of host to absorb nutrients caused by coccidiosis infection. In agreement with our results, the severity of coccidiosis in previous studies showed a significant correlation (r=0.48, P<0.01) with FAMACHA© score (Nurzaty Ewani et al., 2014; Meradi and Bentounsi, 2021). In these studies, they observed FAMACHA© score ranged between 2-3 which is slightly higher than the observed FAMACHA scores in the present study, which ranged between 1.43 (HPN, healthy) to 2.43 (LPN, infected). Another rational explanation for the anemic conditions we observed in infected groups may have caused by the occurrence of *H. contortus* even though it was not determined in the present study. *H. contortus* is the most pathogenic blood-sucking gastrointestinal nematode in ruminants and it causes anemic condition and also contributes to the severity of coccidia. This explanation is supported by a study where they reported anemic conditions in lambs and goat kids infected with both Eimeria spp. and *H. contortus* (Acharya et al., 2020).

Rumen VFA

Rumen VFA profile is known to be altered in response to the different ratios of forage to concentrate due to the changes in nutrient supply. However, other than isobutyrate, no differences of VFA concentrations or molar proportions in response to the plane of nutrition and health status were observed in this study. This is not particularly surprising because animals were fed similar diets, differing only by the mass of concentrate allocated daily. The lack of dietary influence on VFA might reflect the ability of the rumen and the animal to adapt to appropriate dietary concentrate: forage ratios through the self-adjustment of forage intake under ad libitum access to forage. In agreement with our results, previous studies reported no changes in total VFA of Tibetian sheep fed with different ratios of forage and concentrate (Liu et al., 2019a). In ruminants, total VFA concentrations may be as low as 30 mM or be in excess of 200 mM but is typically between 70 and 130 mM (Dijkstra et al., 2005). In our data we observed total VFA concentrations across groups were closer to the lower end. These data suggest that altering the plane of nutrition, within the bounds of this study, while maintaining ad libitum access to hay, supported fairly consistent rumen VFA conditions. Importantly, this should not be conflated with similar energy supplied by VFA from the different planes of nutrition, because rumen VFA concentrations do not take into account production, absorption, and interconversion of VFA, and are considerably influenced by rumen fluid pool size and dynamics. As such, these data are best interpreted to support a consistent form of fermentation among the two planes of nutrition.

Effect of coccidiosis on rumen fermentation and VFA profile is not extensively studied mainly because the negative impacts localized in the small and large intestines (Chartier and Paraud, 2012; Etsay et al., 2020). Coccidiosis from the *Eimeria spp*., results in destruction of the epithelial cells of the intestine hence the coccidia infection strongly interacts with the digestive microflora. A previous study reported a significant change in digestive microflora in goat kids where they observed progressive reduction of the Gram-positive population from 84% pre-infection to 24.3% after the onset of diarrhea. On the other hand, the Gram-negative population was conversely increased from 16% pre-infection to 75.7% after diarrhea (Mohammed et al., 2000). Therefore, we can assume that infection of coccidiosis also has the capacity to alter rumen microflora of lambs. This mechanism supports the lower concentrations of total VFA and individual VFA associated with coccidiosis infected groups. Lower concentrations may also reflect dysregulated fluid dynamics, which are consistent with GI infection.

We did not observe differences in molar proportions of individual VFA across groups, but the low butyrate molar proportions in all groups is noteworthy. Moreover, the interaction effect of nutrition and coccidiosis infection had a tendency towards altering butyrate concentration ($P = 0.058$) resulting lowest concentration in LPNC group, and both observations suggest modifications to butyrate in the rumen. Alterations in butyrate production make sense given its role in functional development of rumen epithelium (Lin et al., 2019; Liu et al., 2019b) and also in lower GI tract (Górka et al., 2011; Kowalski et al., 2015). Studies have reported that increased concentration of butyrate is highly correlated with the enlargement of the ruminal epithelium absorptive surface area (Malhi et al., 2013). We can speculate that the interactive effect of health and nutrition across all the groups suppressed the activity of certain rumen microorganisms which are responsible for producing butyrate, hence low concentration and molar proportion of butyrate. It is possible that these shifts may also confer under-development and reduced functionality of the rumen and intestinal epithelium cells. This hurdle of poor rumen functionality possibly lowered feed utilization by lambs leading to the low weight gains observed. One can argue that the above concept can be reverse engineered; impaired health directly suppressed the development and functionality of the rumen epithelium and limited the utilization of butyrate by the cell wall hence accumulating more butyrate in the rumen. The observed numerically higher butyrate molar proportions in coccidiosis infected groups support the possible importance of this feedback in driving animal physiology. The same explanation of acid accumulation and hence slowing down the synthesis of VFA can be applied to the other VFA concentration data as well (Monteiro and Faciola, 2020). Nevertheless, the lack of confirming measurements and prevailing lack of statistically significant differences among molar proportions precludes definitive discussion on these linkages. Future work should further explore the role of butyrate dynamics and epithelial function during GI parasite infection to more thoroughly explore these concepts.

CONCLUSION

This study sought to investigate the effect of plane of nutrition and coccidiosis infection on finisher lamb growth performance, FAMACHA score, and rumen volatile fatty acid profile. Plane of nutrition did not have an impact on any of the response variables considered. Health status showed a significant effect on FAMACHA score and concentrations of isobutyrate and tendency towards total VFA, acetate, and butyrate suggesting both or either, alteration in rumen microbial profile or rumen epithelium as explanations. An interaction between the plane of nutrition and health status was identified for butyrate concentrations. Overall, this result leads us to assume that changes in nutritional plane could have an impact on growth performance but in our study the effect of health status were more prominent suggesting that it is challenging to overcome the effect of suppressed immunity through nutritional strategies.

In future studies, it would be beneficial to focus on the effect of coccidiosis infection during rumen and overall gut development (preferably lambs below 3 months old age) and consequently how it will impact overall rumen function later in life. This will be helpful to develop clinical and nutritional interventions to support animals that have been exposed to coccidiosis and other related infections.

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Tables

Table 6-1. Chemical composition of grass hay and commercial concentrate fed to lambs during the experiment.

Item	Grass hay	Commercial concentrate*
Dry matter, %	93.9	91.6
Composition, % of DM		
Crude protein	7.70	15.7
NDF	69.0	12.3
ADF	39.1	6.70
Lignin	4.80	0.92
Starch	3.10	49.5
Ash	5.74	8.03
Calcium	0.39	1.09
Phosphorus	0.32	0.52
Magnesium	0.27	0.36
Potassium	1.83	0.85

*Ingredients of the commercial concentrate: Processed grain by-products, grain products, roughage products, plant protein products, cane molasses (with propionic acid, sodium benzoate, potassium sorbate as preservatives), calcium carbonate salt, ammonium chloride, lignin sulfonate, soybean oil, sodium selenite, potassium sulfate, vitamin E supplement, magnesium sulfate, vitamin A supplement, vitamin D3 supplement, zinc oxide, manganous oxide, ferrous sulfate, calcium iodate, cobalt carbonate. Medicated for the prevention of coccidiosis caused by *Eimeria ovina, Eimeria crandallis, Eimeria ovinoidallis, Eimeria parva* and *Eimeria intricate* in sheep maintained in confinement.

Lambs in 2 nutritional planes (HPN and LPN) received the diet in Table 6-1 consisted of grass hay (ad libitum) and different levels of commercial concentrate (0.90 kg/animal/day for HPN group and 0.45 kg/animal/day for LPN group).

Table 6-2. Weight gain and FAMACHA score of lambs in response to the plane of nutrition and health status.

Item	HPNH	HPNC	LPNH	LPNC	SEM	P value		
						Nutrition	Health	Nutrition × Health
Total WG, kg	20.3	15.90	16.7	16.8	3.83	0.738	0.482	0.463
ADG, g	87.5	68.8	72.0	72.4	16.52	0.738	0.482	0.463
FAMACHA	1.70	1.93	1.70	2.23	0.311	0.720	0.047*	0.348

HPNH: high plane of nutrition healthy, HPNC: high plane of nutrition coccidiosis infected, LPNH: low plane of nutrition healthy, LPNC: low plane of nutrition coccidiosis infected, WG: weight gain, ADG: average daily gain. Number of lambs included in each group was; HPNH = 7, HPNC = 3, LPNH = 8, LPNC = 6

* $P \leq 0.05$.

Table 6-3. Volatile fatty acid profiles of lambs in response to the plane of nutrition and health status

Item	HPNH	HPNC	LPNH	LPNC	SEM	P value		
						Nutrition	Health	Nutrition × Health
Total VFA, mM	46.5	45.3	47.2	29.0	7.55	0.380	0.085	0.129
Acetate, mM	29.7	28.4	29.4	18.6	5.08	0.418	0.071	0.150
Propionate, mM	11.63	11.46	12.31	7.31	2.02	0.426	0.121	0.147
Butyrate, mM	2.88	3.57	3.18	1.73	0.675	0.357	0.433	0.058
Valerate, mM	0.704	0.760	0.696	0.532	0.144	0.436	0.644	0.354
Isovalerate, mM	0.482	0.397	0.576	0.286	0.131	0.950	0.120	0.376
Isobutyrate, mM	0.799	0.513	0.897	0.592	0.157	0.573	0.037*	0.938
A/P	2.50	2.52	2.50	2.59	0.182	0.484	0.744	0.324
Acetate, %	64.3	64.0	62.5	62.5	2.22	0.516	0.938	0.928
Propionate, %	25.0	25.4	25.9	25.0	1.40	1.000	0.843	0.586
Butyrate, %	5.97	6.69	6.35	6.61	0.759	0.861	0.436	0.710
Valerate, %	1.45	1.73	1.50	1.80	0.397	0.773	0.120	0.959
Isovalerate, %	0.983	0.892	1.2	1.15	0.393	0.645	0.749	0.929
Isobutyrate, %	1.67	1.17	1.84	2.12	0.701	0.558	0.697	0.196

HPNH: high plane of nutrition healthy, HPNC: high plane of nutrition coccidiosis infected, LPNH: low plane of nutrition healthy, LPNC: low plane of nutrition coccidiosis infected, WG: weight gain, ADG: average daily gain. Number of lambs included in each group was; HPNH = 7, HPNC = 3, LPNH = 8, LPNC = 6

* $P \leq 0.05$

GENERAL CONCLUSION

Improving the understanding of complexity of associations and relationships in rumen fermentation is a key component in improving ruminant animal productivity. Knowledge and understanding can be improved through novel analytical approaches to better depict the data and also by developing methods to quantify more meaningful parameters. An example for the latter is to estimate VFA production and absorption dynamics in place of molar proportions or concentrations. Therefore, the major objective of this work was to investigate the potential of novel analytical methods to explore complex associations in ruminant nutrition and rumen environment and to develop a method to estimate VFA production and absorption dynamics. In parallel to the major objective, we also investigated how different degradability of nutrients and health challenges impacted rumen VFA profile.

Chapter 3 evaluated the potential of alternative analytical approaches to complement traditional meta-analysis approaches using mixed models. Although meta-analysis approach has been used widely in animal nutrition, the approach suffers from challenges such as variable selection bias and poor capacity to handle multicollinearity The objective of this study was to leverage two alternative approaches namely, random forest recursive feature elimination (RFE) and additive Bayesian network (ABN) along with mixed-model analysis. Capacity to capture interactions was a consistent strength of both mixed-models and ABN while RFE could identify main effects only indicating poor performance of RFE in explaining complexity of relationships. In most instances mixed models and ABN were in agreement with the important associations and interactions identified. Further, when mixed models are leveraged to explore associations which are not clearly understood from existing models and literature, conducting an ABN may be useful in selecting the best set of variables, main effects and interactions thus facilitating more data driven analysis. As concluding remarks, ABN provides a simplified but intuitive means of exploring holistic associations within a complex, intercorrelated data environment and mixed models provide a tool which is easier to use quantitatively. Therefore, future studies may benefit from the use of both mixed-models and ABN to better explore associations within complex the data both visually and quantitatively.

In Chapter 4, our objective was to leverage 2 network analyses, a frequentist approach (EBIC-LASSO network; ELN) and a Bayesian learning network (BLN) to explore complex associations inherent within the rumen environment. The generated ELN network was undirected and contained feedback loops and the BLN was a directional network representing implied causality of associations. The illustration of ELN is important because it allowed feedback loops which represent the biological systems better and also highlighted important variables within the system. The BLN holds it strength due to its potential to visualize and understand control pathways within complex ecosystems. Both networks were in agreement in identifying prominent variables and identified variables that may be of interest for future research (ex: valerate). A limitation we observed in this study was the mediocre strength and stability of the networks, likely due to the small data set. Despite these limitations, network approaches exhibited the capacity for more robust exploration of mechanism and relationships within the rumen.

In Chapter 5, we characterized rumen VFA dynamics in response to the varying degradability of protein and fiber of meal fed sheep. The objective of this study was to develop a cost effective and efficient screening method using time-series fermentation data to compare fermentation conditions. Concentrations and molar proportions of acetate and butyrate were higher with beet pulp diets while isoacids were more sensitive to timothy hay diets. Results show that VFA production was minimally affected by the different degradability of nutrients, but changes in absorption dynamics were reported. The higher absorption rates were associated with heat treated soybean meal and it was mirrored in the low concentrations of VFA in the rumen correspondents to the heat treated soybean meal diets. Development of such a method will hugely benefit future ruminant nutrition experiments and may be able to be used as an alternative to iso-tope based assessments.

Chapter 6, the final chapter, sought to investigate growth performance and rumen VFA profile of finisher lamb in response to different planes of nutrition and naturally occurring coccidiosis. We observed the changes of isobutyrate concentrations and tendency towards total VFA, acetate, and butyrate in response to the health status. This suggests that coccidiosis infection can alter either or both rumen microbial profile and the function of rumen epithelium. This is an important finding since the studies investigated the effect of coccidiosis on rumen VFA is scarce. Given the importance of VFA as the main energy source to the ruminant animal it is beneficial to understand how parasitic infections such as coccidiosis would influence rumen VFA profile. Future work should attempt to study the effect of coccidiosis infection during rumen and overall gut development and consequently how it will impact overall rumen function later in life. Knowing the detrimental impact of coccidiosis and whether we can overcome it through nutritional strategies would put us in a better position to develop clinical and nutritional interventions to support animals that have been exposed to coccidiosis and other related infections.

The demand for animal source food continues to grow and the ruminant livestock industry is facing the challenge of meeting this demand while maintaining sustainability. Therefore, strategies to improve ruminant animal productivity are vital. Enhancing ruminant animal productivity and efficiency can greatly evolve with the advancement of our understanding of rumen fermentation dynamics. By tradition ruminant nutrition experiment approaches are reductionist whereby only a few variables are considered, and these approaches are time, labor and financial intensive. The first half of the work presented here successfully utilized alternative analytical approaches to explore intercorrelated associations and relationships in complex environments and data sets we often encounter in animal nutrition. Further, the approaches we discussed here illustrate complex environments such as rumen in a holistic manner, overcoming the inherent reductionism up to some degree. Through the alternative experimental approach, we successfully characterize the dynamics of VFA production and absorption in response to different nutrient degradability. With future research we believe that this experimental method can be developed into a feasible and more practical tool to use in ruminant nutrition experiments. Overall, this body of work helps us to advance our understanding of ruminant nutrition and rumen fermentation through holistic approaches and a new experimental method in quantifying fermentation dynamics.