Effects of ruminal nutrient degradability on volatile fatty acid dynamics, ruminal epithelial gene expression, and post-absorptive system

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SCHOLARLY ABSTRACT

This study evaluated degradable nutrient supply effects on VFA concentrations, fluid flux and pool sizes, rumen epithelial metabolic and absorptive genes, and post-absorptive muscle and blood responses. Six ruminally cannulated Holstein heifers (BW = 330 ± 11.3 kg) were used in a partially replicated Latin Square experiment with four treatments consisting of beet pulp or timothy hay and barley or corn grain. Periods were18 d with 3 d diet adaptation and 15 d of treatment. During each period, d 10 to 14 was used for *in situ* nutrient degradation assessment, d 16 to 18 was used for rumen fluid sampling, and d 18 was used for rumen papillae and skeletal muscle biopsies and blood sampling. *In situ* ruminal starch disappearance rate (barley 7.61 to 10.5 %/h vs corn 7.30 to 8.72%/h; $P = 0.05$) and extent of fiber disappearance (timothy hay 22.2 to 33.4 % DM vs beet pulp 34.4 to 38.7 % DM $P = 0.0007$) differed significantly among diets. Acetate ($P = 0.02$) and isovalerate ($P = 0.008$) molar percentages (% mol) were increased by timothy hay, but propionate ($P = 0.06$) and valerate ($P = 0.10$) molar percentages were decreased. Corn increased propionate ($P = 0.02$) and valerate ($P = 0.049$) molar percentage, but decreased butyrate ($P = 0.04$) molar proportion. Fluid volume and fluid passage rate, and individual VFA pool sizes were not influenced by diet ($P > 0.05$). Four epithelial genes, two metabolic and two absorptive, had increased expression on timothy hay diets ($P < 0.15$). Blood acetate concentration was influenced by treatment ($P = 0.067$) but no other blood metabolites were. Skeletal muscle metabolic rate was significantly increased on corn diets ($P = 0.023$). The results of this study provide a whole-system snapshot of how the rumen environment changes on diets differing in nutrient degradability and how the post-absorptive system adapts in response.

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GENERAL AUDIENCE ABSTRACT

Over the last 50 years, dairy cattle have been bred to optimize milk production to meet growing population demands for milk and dairy products. The world population continues to grow and is projected to reach 9.7 billion people by 2050. Because of this growing population, there is an overwhelming need for dairy nutritionists to optimize the conversion of human inedible fibers into human edible food. The ruminant animal accomplishes this conversion through microbial fermentation of feedstuffs into volatile fatty acids (VFA), which account for approximately 70% of total energy available for meat, milk, and fiber production. Because rumen fermentation is a complex biochemical system, it is influenced by myriad factors including the substrate provided, the pH of the environment, and the absorptive and metabolic capacity of the rumen wall, among others. Although we understand how diet influences individual aspects of rumen fermentation, few studies have concurrently evaluated how diet influences the rumen chemical environment, the epithelium, and the resulting shifts in post-absorptive metabolism. Our study sought to understand the impacts of feedstuffs with different expected ruminally available starch and fiber supplies on these aspects of ruminant physiology. Six ruminally cannulated Holstein heifers were fed four different diets which used either beet pulp (low fiber ingredient) or timothy hay (high fiber ingredient), and ground corn (low starch ingredient) or ground barley (high starch ingredient). Heifers were fed each diet for a period of 18 days. From day 10 to day 14 of the period, nutrient degradability was assessed by incubating bags of feed in the rumen and conducting feed analysis after removed from the rumen. During the last four days of each period, rumen fluid samples, blood samples, muscle biopsies, and rumen papillae biopsies were collected. Feed analysis indicated that the starch sources differed in

degradation rates (i.e. the speed of degradation) and fiber sources different in extent of rumen degradation (i.e. the percentage of feed degraded). Timothy hay caused greater concentrations of Total VFA, Total branched-chain VFA, acetate isobutyrate, and isovalerate. Timothy hay caused greater molar proportions of acetate and isovalerate. Corn caused greater molar proportions of propionate and valerate when barley caused greater molar proportions of butyrate. Rumen papillae biopsies were used to evaluate gene expression. Out of 14 genes, four were impacted by diet. Two rumen transporters responsible for the absorption of VFA had greater expression when animals were fed timothy hay diets versus beet pulp diets. Two metabolic genes also had greater expression due to timothy hay. The changes of both absorptive genes and metabolic genes is likely connected to the increased presence of VFA in the rumen. Lastly, blood acetate was increased, but there was not a specific ingredient or combination that caused the change. These results provide an overall snapshot of rumen fermentation characteristics and how changes in the rumen affect other biology.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

There is an overwhelming need for animal product producers to find the most humane, economical, and environmentally efficient strategies to produce more meat, milk, and co-products (i.e. cheese, butter, etc.). Factors driving the need for increased efficiency are: the increasing global population expected to reach 9.2 billion people by 2050 (Thornton, 2010); increasing pressure for animal agriculture to be more environmentally friendly; and consumers being further removed from agriculture but still curious about how their food is produced. In livestock production, efficiency is commonly defined as the ratio between every unit of feed and every unit of product (meat, milk, etc.) produced (Hanset et al., 1987). This ratio is also known as the Feed to Gain (F:G) ratio (Hanset et al., 1987). An inefficient animal would consume more feed for every one unit of product produced (e.g. F:G 2:1). An efficient animal would consume less feed for every one unit of product produced (e.g. F:G 1.5:1). By increasing ruminant feed efficiency, farmers can feed less and either maintain or increase production. Increasing feed efficiency also can allow for feeding more animals per unit of land. Ruminant feed efficiency is a function of genetic (Hardie et al., 2017) and environmental (Hooven et al., 1968), and individual animal factors (e.g. age, parity) (Hooven et al., 1968). Optimizing supply of volatile fatty acids (VFA) is one nutritional way to increase ruminant feed efficiency.

Volatile fatty acids are the end products of microbial fermentation and account for 70 to 80% of energy available to the ruminant (Bergman, 1990). Because VFA are an output of microbial fermentation, optimizing VFA supplies to the animal requires considering the substrate provided for fermentation, the chemical environment of the rumen, the microbes available for fermentation, and the capacity of the rumen fluid and epithelium to remove end-products of fermentation. Historically, work has focused exclusively on rumen VFA concentrations as a way

to represent fermentation; however, these concentrations are not the most accurate way to describe shifts in the rumen environment associated with fermentation (Hall, 2015). The rumen is a dynamic, kinetic ecosystem with constant inputs and outputs. To describe VFA as concentrations is an incomplete characterization because multiple factors influence the presence or absence of VFA in the rumen. To more fully understand how to optimize VFA supplies, there must be a conversion from the use of VFA concentrations to VFA pool size. Pool size describes the amount of VFA as a function of individual VFA concentrations, rumen fluid volume, and fluid flow (entry and exit) (Hall, 2015).

When characterizing fermentation responses to dietary changes, it is also critical to evaluate the responses of the rumen epithelium. A healthy rumen epithelium is critical for VFA absorption and metabolism. There are multiple short chain fatty acid, electrolyte, proton, and other transporters imbedded in the rumen papillae (Aschenbach et al., 2009; Kramer et al.,1996), which drive majority of ruminal absorption (Gabel and Sehested, 1997). The propensity of transporter adaptation to different VFA supplies, diets and possibly detrimental rumen conditions has partially been described in the literature but warrants further investigation because fermentations with different characteristics often yield unique and, at times, inconsistent responses. An example of these unique responses associated with fermentation environment can be noted in Steele (2011) when animals had structural epithelial adaptations ultimately impacting the expression of different transporters due to intense grain feeding.

The objective of this study to investigate effects of nutrient disappearance on VFA dynamics and epithelial gene expression using calculated VFA pool size and fluid mediated flux as more accurate indicators of VFA dynamics. Chapter 3 describes a different approach to enhance feed efficiency by understanding VFA dynamics. Treatment diets were formulated

based off predicted rumen disappearance to altered fiber and starch sources based on assumed microbial fermentation patterns. The procedural design bridges identified gaps by using a rumen fluid marker to measure rumen volume and passage rate. Samples were taken every hour for 21 to 30 hours to measure VFA concentration and marker degradation over time. Fluid-mediated VFA flux and pool size were calculated from VFA concentrations and rumen fluid dynamics results. By integrating rumen fluid measurements with VFA concentrations, we gain a more robust understanding of how VFA and changing in the rumen.

Another factor influencing an animal's feed efficiency is their maintenance requirements, which are quantified by measuring basal metabolic rate. Historically, basal metabolic rate is measured using metabolic chambers, which are able to measure the amount of O_2 consumed by an animal and the carbon dioxide produced. Due to facility expense, small sample size, pressure for quantifying greenhouse gas emissions, and amount of labor to conduct a study using metabolic chambers, this method is now less common for basal metabolic rate quantification. However, to better equip an animal to be more efficient, there is a need to understand variation of individual animal maintenance requirements. To avoid using metabolic chambers, a common cell culture assay designed to measure metabolic activity was tested as a way to stratify animals based on skeletal muscle metabolic activity. This assay was adapted from a zebrafish embryo protocol (Williams and Renquist, 2013; Renquist et al., 2016). The objective of this experiment, described in Chapter 2, is to test the repeatability, practicality, and sensitivity of the novel assay. The application of the common cell culture assay to measure skeletal metabolic rate may be a way to reduce the expense and small sample size limitations of metabolic chambers, despite the small sample size shown in Chapter 2. Additionally, after further research is conducted with

larger sample sizes, the assay could be useful for broad-spectrum, low-cost phenotyping of feed efficiency.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

Animal products have been used habitually and routinely in lifestyles for centuries (Larson, 2003). Meat, milk, and respective dietary by-products (i.e. cheese, butter, etc.) have high nutrient to calorie ratios, meaning that they help consumers to meet vitamin and mineral, protein, and essential fatty acid requirements without exceeding dietary energy requirements (White and Hall, 2017). Despite market cyclicity and surplus or rationing of goods, animal products remain in demand (Gerber et al., 2013). Demand for animal products is expected to double in developing countries due to rapid population growth between present and 2050 (Thornton, 2010). Increasing ruminant feed efficiency is one strategy to meet this growing global demand without compromising resources and the environment (Capper and Bauman, 2013). A promising strategy to increase ruminant feed efficiency is to optimize the profile and supply of energy sources, i.e., volatile fatty acids (**VFA**), available to the post-absorptive system. Different energy sources (fat, VFA, sugar) are used for different metabolic processes (production of milk fat, protein, lactose, among others) with different energetic efficiencies (Baldwin, 1968). These differences in efficiency are because of the different metabolic pathways (i.e. glycolysis, oxidation of pyruvate, etc.) needed to convert a substrate to the desired end product. The theoretical energy conversion efficiencies for acetate, propionate, and butyrate are shown in Table 1. The differences in theoretical energy conversion efficiencies suggests an opportunity to optimize post-absorptive efficiency if optimal supplies of each substrate could be delivered from the pre-absorptive system with a high degree of certainty.

Unfortunately, manipulating VFA supplies absorbed from the rumen is a complex task. First, there is a major gap in our knowledge of how different substrates or dietary ingredients influence VFA production, absorption, and interconversion because relatively few studies (e.g., Sutton et al., 2003; Markantonatos et al., 2008) have been specifically conducted to evaluate

VFA absorption. A much larger body of literature has evaluated how diet influences VFA concentrations (e.g. Sutton et al., 2003). However, recent work by Hall et al. (2015) discussed that VFA dynamics are a function of rumen fluid volume and fluxes, as well as VFA pools, which are determined by VFA production, interconversion, and absorption. The work of Hall et al. (2015) identifies a need to quantify VFA by considering the rumen ecosystem as a whole rather than focusing exclusively on concentration.

The last significant gap in our understanding is how the rumen epithelium responds to different fermentation environments, perhaps influencing the capacity of the epithelium to absorb and metabolize VFA. The epithelium consists of proteins responsible for VFA transport (Gabel and Sehested), ion exchange (Gabel and Sehested, 1997), and nutrient metabolism (Bugaut, 1987). Although studies have determined how genes encoding these proteins shift in response to vastly different fermentation environments, such as acidosis (Steele et al., 2011) or different stages of life (Steele et al., 2015), few studies have concurrently evaluated shifts in rumen fermentation and the corresponding responses of the rumen epithelium. More data on these interactions will allow us to understand the nature of rumen adaptations and how we can manipulate the rumen environment and absorbed VFA profiles in order to increase ruminant feed efficiency, decrease feed costs, and preserve rumen health.

Physiology of Digestion

Mechanical and chemical digestion begins at the mouth of ruminant and monogastric species with chewing and saliva release (McDonald et al., 2011). Saliva moistens the feedstuff to assist with travel down the esophagus to either the stomach of monogastric species or the reticulorumen of ruminants (McDonald et al., 2011). Saliva also provides phosphorus, and buffers that will help maintain stomach or rumen pH (McDonald et al., 2011). The two types of

digestive systems (monogastric versus ruminant) start to differ at the stomach and reticulorumen. In monogastric species, the diet is chemically and mechanically broken down by enzymes and stomach contractions. In ruminants, the diet is filtered and fermented in the reticulorumen. The rumen and omasum have productive and absorptive functions (McDonald, et al., 2011). The rumen and omasum produce VFA, and also absorb water, ammonia, VFA, and electrolytes (Dijkstra et al., 2005). The rumen differs from the omasum due to fermentative capabilities. The abomasum functions similarly to the monogastric stomach by secreting enzymes and mechanically mixing the digesta as that digesta continues towards the small intestine. After forestomach digestion, digestion is relatively similar between both types of digestive systems. However, one difference is the fermentative capabilities in the large intestine. Some monogastric species, like hind-gut fermenters (e.g. horses, rabbits), have substantial fermentative capabilities in the large intestine and cecum, but most monogastric species have limited fermentation capabilities in the hind gut (McDonald et al., 2011).

Nutrient Degradation

Nutrient degradation or disappearance refers specifically to the breakdown of feedstuffs by microbial fermentation, typically occurring in the rumen or large intestine (McDonald et al., 2011). Feedstuff complexity and dietary characteristics are the primary determinants of the location of digestion in the gastrointestinal tract. For example, in ruminants, total tract digestibility of starchy feedstuffs is greater than 95% (Tucker, et al., 1968; Cerrilla and Martinez, 2003) with 60 to 75% being degraded in the rumen (Patton et al., 2012). Total tract fiber digestibility is highly variable due different feed stuffs. An example would be corn silage-based diets, which vary between 20 to 60% NDF total tract digestibility (Goeser, 2008). The rumen is responsible for 90 to 95% of total NDF degraded (Huhtanen et al., 2010). Remaining fibrous

feedstuffs are degraded in the large intestine, which functions similar to the cecum of hind-gut fermenters.

There are two degradation parameters to describe disappearance of nutrients-degradation rate and extent of degradation (Hackmann et al., 2010). Degradation rate describes the speed of nutrient disappearance over time (McDonald et al., 2011). Graphically, rate of degradation is determined by the slope (Figure 1). Extent of degradation describes the percent of feedstuff degraded in the rumen (McDonald et al., 2011), and is demonstrated graphically by the "Y" value (Figure 2). Both rate and extent are dependent upon the plant cellular composition of the feedstuff but in different capacities (Moore and Jung, 2001). Plant cellular composition is driven by plant maturity and growing temperature (Nelson et al., 2007). For example, a fibrous feedstuff growing at a warmer temperature contains more hemicellulose and lignin in comparison to an immature plant (Nelson et al., 2007). Additionally, there is an increased build-up of lignin in forage plants that grow at warmer temperatures (Chatterton et al., 1989).

Degradation parameters can also be affected by the methodological technique used to quantify the rate. *In vitro* techniques tend to underestimate degradation rate in comparison to *in situ* techniques because of variable individual animal rumen characteristics and type of diet (Dijkstra et al., 2005). The *in situ* technique provides a representative rumen environment, but is also not the gold standard because results vary with bag placement in the rumen and sample size to bag ratio, among other factors (Liebe et al., 2018). If pores are too large, microbes have greater access to the feedstuff causing an overestimation of degradability, and underestimation can result from small pore size (Dijkstra et al., 2005). The best option to measure ruminal degradability is through the use of a marker and duodenal cannula or omasal sampling (Kozloski, et al., 2014), though this method is more intensive, both in terms of animal use and economics.

Rate and extent of degradation are also dependent upon the processing method of the feedstuff. Forages are normally fed as hays (long-stem) or silage (shorter chop length). Grasses and hays can be further chopped or pelleted to accommodate smaller particle size (Cash et al., 1998). Xu and Harrison (1996) discussed that commercial grains and different by-product feedstuffs had the greatest variations in degradation parameters of several feed classes tested. Normally, these feedstuffs are further processed (ground, pelleted, etc) to increase availability of nutrients for degradation (Theurer, 1986). Pelleting requires crushing, grinding, and molding the feedstuff into a new, pelleted form, typically under high heat and pressure. The grinding causes the physical breakdown of the cell wall allowing for increased access to cell wall components and nutrients. Steam can also be applied either during processing (i.e. steam-rolled corn) or may be the only processing method (i.e. steam flaked corn). Many studies have investigated digestibility between different processing methods of feedstuffs in all livestock species (e.g., Yu et al., 1998; Alvarado et al., 2009). Yu et al. (1998) investigated five different corn processing methods effects on milk yield and components found 1) milk yield, milk components, and nutrient utilization differed due to the type of processing method and 2) depending upon farm goals, a specific type of corn processing may be better. Additionally, feed efficiency, production, and diet cost associated with changes in digestibility due to different processing methods has been quantified (e.g., Yu et al., 1998; Alvarado et al., 2009; Dozier et al., 2010). Economical differences are of particular importance because of the increased cost associated with a more processed diet (Dozier et al., 2010), however, pelleting could cause greater availability of nutrients.

Biological Mechanisms Driving Degradation Differences

The ability for a feedstuff to be degraded is a function of the surface area available for microbial attachment and the complexity and density of the feedstuff cell wall (McDonald et al., 2011). Not all exposed surface area on a feedstuff is available for microbial attachment (McAllister et al., 1994). Microbial attachment happens in three waves: 1) the primary colonizers, which foster an environment on the feedstuff for the next wave of microbes; 2) the secondary colonizers, which start to create a biofilm on the feedstuff and 3) the general microbial population starts attaching to respective nutrients (e.g., cellulolytic bacteria attach to cellulose) (McAllister et al., 1994). Feed chemical factors that inhibit any phases of this process will likely impair microbial attachment, and subsequently feed degradation (McAllister et al., 1994). Cereal grains normally have small particle size and thus have smaller surface area available for attachment, but due to chemical structure of starches are easily degradable. On the other hand, forages have greater surface area for microbes to attach, but due to cell wall complexity are more difficult to degrade (Lynd et al., 2002).

Some feedstuffs can have similar extent of degradation, but different rates of degradation. Rate of degradation is affected by microbial populations (McAllister et al., 1994), feedstuff characteristics (Hackmann et al., 2010), feedstuff processing (Yu et al., 1998), and gut fill (Osbourn et al., 1981). The speed of degradation can be easily influenced by feed processing because a "step" of the degradation process has been externally accomplished (Offner et al., 2003). Extent of degradation is a product of microbial populations, feedstuff characteristics, and passage rate. In comparison to these other factors, feedstuff processing and gut fill are less important for determining a theoretical maximal extent of degradation because microbial degradation will proceed as long as substrate is available. For example, rolled corn will have a

faster degradation rate than whole corn kernels, but will have the same extent of degradation (Offner et al., 2003)

Feeds with higher particle passage rate have lower rumen degradability because particles are exposed to fermentation conditions for less time, and particles from these feeds are often subjected to more extensive digestion in the abomasum and intestine (Ehle, 1984; Offner et al., 2003). Increased passage rate promotes the movement digesta out of the rumen to be further digested. The enzymatic digestion of the abomasum and fermentative capabilities of the large intestine contribute to improving the total-tract digestibility of feedstuffs that have poor rumen fermentation. Examples of feedstuffs with poor fermentability include those that should normally be processed or that have a complex cell wall structure and rumen microbes can only degrade a fraction of the feed (Offner et al., 2003). Liquid passage rate plays a less important role in degradability in comparison to solid digesta passage rate (Casper et al., 1999). Rumen fluid, as the medium and housing environment of fermentative species, is essential for degradation of nutrients in the rumen and theoretically limited rumen volume (due to high passage rate) could impair degradation; however, this is very unlikely (Casper et al., 1999).

Fiber Degradability

Lactating cow rations are typically 35 to 40% NDF (DM basis), the exact NDF level is dependent upon the amount of starch in the diet and the type of forage (NRC, 2001). Studies have shown that dietary NDF below 25% (DM basis) has adverse effects on milk fat, but does not change milk production (e.g. Clark and Armentano, 1993). Dry matter intake was limited with dietary NDF at 44% with lower producing animals (e.g. 20 kg/d) (Mertens, 1994). The NDF content of the diet may change based on production goals, ingredient availability, and general cow management. The rumen is responsible for 90 to 95% of total NDF degraded (Huhtanen et

al., 2010) and typical rumen fiber degradation rate ranges from 4 to 10 %/h (Varga and Hoover, 1983). Both degradation parameters normally dependent upon the amount of digestible cellulose (Varga and Kolver, 1997).

Fiber is typically analyzed as crude fiber, neutral detergent fiber, or acid detergent fiber. Crude fiber contains true cellulose and insoluble lignin (Jung, 1997). Neutral detergent fiber comprises cellulose, hemicellulose, and lignin (Van Soest et al., 1991), while acid detergent fiber contains primarily cellulose and lignin (Minson, 2012). Hemicellulose is classified as lowly-digestible and lignin as indigestible by rumen microbes (Van Soest, et al., 1991). These compounds are challenging to digest because of covalent bonding between hemicellulose and lignin (Chesson, 1993), complexity of chemical structure, and the degree of branching of hemicellulose molecules (Niwińska, 2012). Cellulose in the cell wall, however, is not chemically bound to hemicellulose or lignin, but is chemically bound to individual cellulose molecules though beta-acetal linkages (Altaner et al., 2014).

Fibrous feedstuffs consist of primary and multiple secondary cell walls (Alberts et al., 2002, Pérez et al., 2002). Each cell wall is comprised of an external-most lignin matrix, a middle hemicellulose band, and inner-most cellulose fibril (Pérez et al., 2002). The cellulolytic fibril consists of individual glucose units covalently bonded, and the individual chains are linked by hydrogen bonds (Altaner et al., 2014). Hydrogen bonding is known to strengthen cellulose particularly during stretching or straightening (Altaner et al., 2014). Variability in ruminant cellulose digestion is not necessarily due to the strengthening of cellulolytic hydrogen bonds, but can be explained by association (not bonding) with lignin (Altaner et al., 2014). Because the poorly digestible cell wall components are the most external component of the cell, it is difficult for rumen microbes to penetrate to begin digestion. Processing forages and chewing create

physical breakdown of the cell wall causing easier access to cellulose, but the same processing method for all ingredients is not best because chemical composition is not altered uniformly (Osbourn et al., 1981).

Another factor impacting extent of ruminal fiber degradation and rate is dry matter intake (Van Soest, 1967; Hoover, 1986). Several studies (e.g., Osbourn et al., 1981; Hoover, 1986) have indicated that fiber digestibility is decreased at a higher level of feeding due to increased gut fill leading to slower particle passage rate. Understanding the relationship between fiber digestibility and intake is an ongoing process, but is of interest because animals fed on a high plane of nutrition to support milk production or growth most likely have decreased fiber digestibility due to associative effects with high energy feedstuffs (Mould and Ørskov 1983; Firkins et al., 2001)

Mould and Ørskov (1983) discussed potential reasons behind the negative connection between fiber digestibility and an increase in starch in the diet. These reasons focus on ruminal pH depression and a decrease in cellulolytic bacteria population to accommodate starch digestion (Mould and Ørskov, 1983). Shaver and colleagues (1988) investigated NDF digestibility and passage rate, and believed a depression in NDF fractional rate was due to a shift in the rumen environment to accommodate starch digestion as Mould and Ørskov (1983) had explained a few years prior.

Ruminants are able to digest human inedible fibrous material (grass, hays, silage, some by-products) because of microbial fermentation. Rumen responses to different fiber cell wall densities and complexities (nutrient degradability) requires further study to better understand how fiber in individual feeds degrade and how fiber interacts with other nutrients or aspects of the rumen environment.

Starch Degradability

Starch is comprised of two main polysaccharides-amylose and amylopectin. Amylose is an unbranched starch molecule comprised of alpha-D-glucose linked by glycosidic bonds (Svihus et al., 2005). Amylose accounts for 25% of starch molecules; amylopectin accounts for 75% (Lafiandra et al., 2014). Amylopectin is similar to amylose, but is branched due to glycosidic bonds attaching carbon 4 and 6. The non-linear structure of amylopectin causes it to be more readily digestible than amylose (Svihus et al., 2005). Because the amylose and amylopectin contents of a feedstuff can differ, the ratio of amylopectin to amylose is one way feedstuff degradability can be assessed in vitro. Svihus et al. (2005) discussed the variability of the amylose and amylopectin ratio within commonly used cereal grains.

Svihus et al. (2005) also discussed the impacts of processing methods on starch digestibility and utilization and concluded that due to starch layer organization, abundance of amylose, and fat and protein physical barriers, uniform processing methods would be inappropriate. Starch feedstuff processing has evolved to include grinding, mashing, pelleting, heating, and water treating (Svihus et al. 2005) allowing for specific plant processing to accommodate individual starchy feedstuff differences. As discussed earlier, specific processing also allows for optimal digestibility of a starchy feedstuff (steam-rolled versus steam-flaked versus only rolled; Yu et al., 1998).

The majority of research connecting nutrient digestion and VFA has centered around starch due to the nutrient density and composition of starch feedstuffs (Dijkstra et al., 2005). Cereal grains, classified as starchy feedstuffs, are energy dense (Lafiandra et al., 2014). Energy dense ingredients are used in lactating cow rations to meet energy demands for maintenance, gestation, growth (youngstock), and milk production. Starchy ingredients provide an advantage

over fibrous feedstuffs when considering rumen degradation rate. Offner et al. (2003) reported that cereal grains could have a starch disappearance rate as high as 20%/h when fibrous feedstuffs could have a starch degradation rate as low as 3%/h. A faster rate of degradation demonstrates a feedstuff more available to rumen microbial breakdown that most likely contains less amylose, as discussed earlier. However, a rapid rate of degradation can be described as a disadvantage due to the well-researched inverse relationship between rapidly fermentable carbohydrates and ruminal pH depression (Mould and Ørskov, 1983). Drastic rumen pH depression greatly alters rumen microbial populations (Hook et al., 2011), rumen epithelial integrity (Steele et al., 2011), and rumen electrolyte homeostasis causing poor or impaired digestion, absorption, and utilization of nutrients (Aschenbach et al., 2011). During severe pH depression for a prolonged period, reduction in dry matter intake and subsequently a decrease in milk production can also occur. This concept stems from Firkins (2002) that higher producing animals who normally have higher DMI are more susceptible to ruminal pH depression. When animals consistently consume rapidly fermentable carbohydrates, more VFA are produced causing an accumulation of acids (Stone, 2004) and possibly protons (Aschenbach et al. 2011) ultimately depressing rumen pH. Animals will most likely consume less or consume the forage-based portion of the ration causing milk production to suffer (Beauchemin, 1991). The connection between rapidly degraded carbohydrate sources and pH depression has led to the assumption that starch is a driving force behind subacute ruminal acidosis (SARA) and acute acidosis in cattle. Dietary inclusion of rapidly degradable starch sources requires ruminant nutritionists to balance meeting energy requirements for combined maintenance and production with the goal of maintaining rumen health.

Extent of ruminal starch degradation ranges from 60 to 80% meaning majority of starch is degraded in the rumen (Harmon, 2009; Patton et al., 2012). Majority of starch should be degraded in the rumen because an influx of starch to the large intestine could compromise intestinal integrity causing leaky gut (Kvidera et al., 2018). Accordingly, starch total tract digestibility ranges from 85 to 95% of total starch content (Patton et al., 2012).

Current Knowledge of Starch and Fiber Extent and Rate of Disappearance

Offner et al. (2003) reviewed ruminal starch degradation and provided significant information describing the factors that regulate degradability and degradation rate. We currently know that nutrient extent and rate of disappearance are affected by similar factors (feedstuff chemical complexity, microbial populations, etc.), but not exactly the same factors because a feedstuff can have different degradation rates and similar extent of degradation (Offner et al., 2003). A more recent review by Guiberti et al. (2014) discussed similar information as Offner et al. (2003), but discussed the need for enhanced plant breeding programs rather than animal interventions to increase starch digestibility. Possibly a combination of plant breeding and animal intervention could optimize starch digestibility.

Total tract fiber digestibility ranges from 40 to 70% total NDF (Buxton and Redfearn, 1997). Variability in fiber digestibility is due to the type of fiber (concentrate versus forage fiber), leaf to stem ratio of an individual forage, and rumen microbial populations (Buxton and Redfearn, 1997). Additionally, there has been work describing changes in processing methods or conservation methods to render fiber more degradable (Sousa et al., 2014). More recently, particle size and chewing times have played a significant role in understanding fiber digestibility. Because Mertens et al. (1997) described physically effective neutral detergent fiber (peNDF), papers like Zebeli et al. (2012) conducted experiments to gain data further understanding fiber

digestibility as a function of particle size and the portion of fiber contributing to the forage mat. Then, White et al. (2017) took peNDF a step further by describing physically adjusted neutral detergent fiber (paNDF) as a function of peNDF, particle size and individual feed NDF. The authors were able to connect paNDF to individual animal parameters such as: dry matter intake, rumen pH, and rumination time. Future work would include gaining more animal data regarding peNDF and paNDF.

Protein Degradability

Two general classifications of protein in ruminant diets include rumen undegradable protein (RUP) and rumen degradable protein (RDP). Rumen undegradable protein is not degraded in the rumen and bypasses directly to the intestine (McDonald et al., 2011). Rumen degradable protein provides a substrate for microbial protein synthesis (McDonald et al., 2011). Additionally, the ratio between RDP and RUP has been linked to net milk production, feed efficiency, and nitrogen efficiency (Savari et al. 2018). Savari et al. (2018) found that animals consuming a high RDP:RUP diet produced more milk with no difference in dry matter intake. Accordingly, animals with high RDP:RUP demonstrated greater feed efficiency and higher apparent N efficiency (Savari et al., 2018). The relationship between increasing RDP:RUP and greater feed efficient animals warrants further investigation because the ratio could provide another avenue to increase feed efficiency.

Microbial protein is produced by microbes consuming ATP causing microbial growth (Hackmann and Firkins, 2015). Historically, substrates for microbial protein production have been fibrous feedstuffs with poor protein content (accessible protein) and non-protein nitrogen sources (Dewhurst et al., 2000), but recent land competition and increased costs of different feedstuffs has caused a shift in the type of substrate used to produce microbial protein (Matassa

et al., 2016). Now energy dense carbohydrates are fed to generate more ATP through carbohydrate excess for microbial protein synthesis (Hackmann and Firkins, 2015).

Microbial protein production is of particular importance because 60 to 85% of amino acids that reach the small intestine are derived from microbial protein (Storm et al., 1983), and half of microbial protein derived amino acids are absorbed and utilized by the animal (Agricultural and Food Research Council, 1992). However, microbial protein synthesis does not produce adequate amounts of amino acids to meet requirements and rumen microbial enzymes degrade amino acids unless protected (Chalupa, 1975). Thus, supplementation of ruminally protected amino acids destined for post-ruminal degradation (i.e. RUP) is common practice to meet amino acid requirements.

According to Erasmus et al. (1994), the amino acid profiles of the RDP fraction had greater abundance in the post-absorptive system suggesting ruminal degradation has greater impact on post-absorptive amino acid profiles in comparison to intestinal protein degradation (Erasmus et al., 1994). Despite the positive relationship between RDP and microbial protein production, if there is over-supplementation of RDP, then large amounts of ammonia are ruminally produced (Savari et al., 2018). Ruminal production of ammonia can be absorbed, converted to urea, and excreted in urine and manure ultimately increasing overall nitrogen excretion (Muck, 1982).

Urea recycling is one biological way to reduce nitrogen excretion while meeting amino acid requirements. Urea recycling salvages nitrogen in the form of urea and transports it back to the rumen to be used for microbial protein synthesis (Lapierre and Lobley, 2001). Lapierre and Lobley (2001) discussed two major options to mitigate the N losses and environmental impact: 1) by decreasing amino acid catabolism and ammonia absorption by providing and 2) increasing

the conversion efficiency of urea nitrogen to bacterial protein, which are normally measured by isotope dilution techniques through urine collection. This allows for the origin of nitrogen atoms to be traced to determine if they were recycled or not (Lapierre and Lobley, 2001).

General Introduction to VFA

Eleven VFA are commonly discussed with respect to rumen fermentation: acetate, propionate, butyrate, formate, valerate, hexanate, heptanate, isovalerate, isobutyrate, and 2-methylbutyrate. These VFA differ by the number of carbon atoms and shape of the carbon chain: straight versus branched (Bergman, 1990). Acetate (2 carbon chain), propionate (3 carbon chain), and butyrate (4 carbon chain) comprise 95% of total ruminal VFA supply (Bergman, 1990). Out of the 95%, ruminal acetate typically comprises 70%, propionate 20%, and butyrate 10% (France and Djikstra, 2005).

Volatile fatty acids are produced by microbial fermentation, but due to specie digestive and metabolic differences, fermentation takes place in different locations of the gastrointestinal tract. In humans and monogastric animal species VFA are produced in the large intestine, particularly the cecum and colon (Fleming and Arce, 1986). In ruminant species, VFA are mainly produced in the rumen. Location of VFA production in ruminants was first investigated by Elsden et al. (1946) who discovered that ruminal VFA supply was greatest; however, there was substantial VFA supplies in the large intestine of ruminant species. Differences in VFA supply based on gastrointestinal tract location were further confirmed by subsequent studies (Faichney, 1968; Goodall and Kay, 1965; Siciliano-Jones and Murphy, 1989). Production of VFA is driven by the specific microbial population present in the rumen or large intestine (Bergman, 1990). Slight changes in diet alter microbial populations and subsequently VFA supplies. Additionally, Bergman (1990) discussed nutrient disappearance is proportional to VFA

concentrations at different locations. However, an individual animal's specific microbial population may be more influential than a change in diet (Weimer et al., 1999).

Mechanism of VFA Production

Volatile fatty acids are produced from microbial fermentation in the rumen. Microbial fermentation relies on rumen microbes (bacteria, fungi, protozoa) to attach, destroy, and breakdown feedstuffs (McDonald et al., 2011). Destruction of the plant allows for the different chemical components, cellulose, hemicellulose sugars, and starches, to be further broken down into glucose, a simple sugar (McDonald et al., 2011). The first step in microbial fermentation is glycolysis when glucose is converted to pyruvate. Pyruvate can be broken down into many end-products through the tricarboxylic acid cycle, and the population of microbes determines which end-product route pyruvate will take (McDonald et al., 2011). Microbial populations are influenced by diet and, in some microbial measurements, breed (Gleason and White, 2018).

Microbial fermentation is an energy consuming and energy producing process causing energy to convert between different forms (Hobson and Stewart, 2012). One such conversion is the transfer of chemical energy into gaseous energy in the form of carbon dioxide and methane (Hobson and Stewart, 2012). Chemical energy can also be converted into heat energy by microbial fermentation, which partially contributes to reduced dry matter intake during hot seasons or in hot climates (Conte et al., 2018).

Effects of Feed Type on VFA Supplies

The amount and type of fermentation end products (carbon dioxide, VFA, alcohol, etc) are diet-dependent. Murphy et al. (1982) investigated the impact of different nutrient substrates from roughage and concentrate feedstuffs on VFA stoichiometric coefficients through modelling.

Concentrate diets increased acetate and propionate stoichiometric coefficients in comparison to roughage diets. In contrast, roughage diets produced higher stoichiometric coefficients of valerate, but butyrate did not differ. Sutton et al. (2003) conducted an animal experiment similar to what Murphy et al. (1982) simulated. Sutton et al. (2003) fed a "normal diet" consisting of 7.8 kg DM/d concentrates and 5.1 kg DM/d hay and a "low roughage diet" consisting of 11.5 kg DM/d concentrates and 1.2 kg DM/d hay. Sutton et al. (2003) measured VFA concentrations, and calculated molar proportions, VFA production, and interconversion through infusing radio-isotope (C^{14}). Sutton et al. (2003) found that acetate and butyrate concentrations were greater on the "normal diet". Propionate and valerate concentrations were greater when animals consumed the low roughage diet (Sutton et al., 2003). On a molar proportion basis, acetate and butyrate were greater when animals consumed the "normal diet", and propionate and valerate were greater when animals consumed the low roughage diet (Sutton et al., 2003). Although VFA concentrations and molar proportions are still accepted and widely reported in the literature, Sutton et al. (2003) also measured rumen fluid dynamics using intraruminal markers and infused isotopically labeled VFA allowing for estimations of VFA production, absorption, and interconversion to be calculated. Some 12 years later Hall et al. (2015) urged for rumen fluid dynamics to be measured in order to have a better interpretation of VFA concentrations and molar proportions, but also to more accurately describe VFA production, absorption, and interconversion.

One similar finding between Murphy et al. (1982) and Sutton et al. (2003) is both reported finding similar changes in propionate. However, the units of estimation or measurement differed between the articles with Murphy et al. (1982) providing simulated estimations of stoichiometric parameters and Sutton et al. (2003) measured VFA concentrations, molar

proportions, and production. Murphy et al. (1982) specifically noted that starch from concentrates was one of the greatest contributors to greater stoichiometric coefficients of propionate. This increase was speculated to be due to a shift in rumen microbes, particularly amylolytic and lactate-utilizing bacteria.

There was conflicting information regarding butyrate results between the two studies. The Murphy et al. (1982) simulation demonstrated changes in butyrate stoichiometric coefficients. Predicted butyrate stoichiometric coefficients associated with fiber are primarily substrate driven when this was not investigated in Sutton et al. (2003). It is also possible that VFA profile shifts discussed in Murphy et al. (1982) are concurrent to shifts in the microbiome.

Mrázek et al. (2006) considered the relationship between fiber and increasing ruminal butyrate presence by investigating butyrate-producing bacteria: *Butyrivibrio* and *Pseudobutyrivibrio*. Interestingly, high fiber diets only significantly impacted *Butyrivibrio,* but did not impact *Pseudobutyrivibrio* enumeration (log n/mL; Figure 2 in Mrázek et al. (2006)). Mrázek et al. (2006) did not measure ruminal butyrate concentrations, molar proportions or production. Paillard et al. (2007) drew connections between phylogenetic position of *Butyrivibrio* and *Pseudobutyrivibrio* and ruminal butyrate through increased ruminal butyrate kinase activity. Paillard et al. (2007) only measured enzyme upregulation to determine increased ruminal butyrate. This method does not accurately describe ruminal butyrate pool size, but the experimental findings reiterate that feed type affects microbial populations.

Like Sutton et al. (2003) finding increased acetate concentrations and molar proportions in response to roughages, there has been further work discussing high forage diets having greater impacts on ruminal acetate and acetate to propionate ratio (Moran, 2005). The information demonstrated in Moran (2005) stemmed from information presented by Mowrey et al. (1999).

Mowrey et al. (1999) discussed switching forage and concentrate-based ingredients to determine the impact on VFA supplies. When animals were fed diets containing high-fiber ingredients ruminal acetate concentration increased. Contrastingly, when animals were fed high-concentrate ingredients, propionate concentration increased. Changes in ruminal acetate and propionate, and acetate to propionate ratio associated with forage to concentration ratio was further supported by Sarwar et al. (1991, 1992).

Effects of Substrate Source on VFA Supplies

Metabolic pathways require a constant supply of substrate. If substrate become limiting, then severe adverse downstream effects can occur (e.g. lack of electron transporters for electron transport chain) (McDonald et al., 2011). If there is excess substrate, the metabolic pathway is flooded and will typically respond by changing the rate of the metabolic process through enzyme upregulation or the substrate will go elsewhere, if possible (McDonald et al., 2011). Additionally, the type of substrate fed to a metabolic pathway elicits different responses. A prime example is type of feed chemical compound (cellulose, hemicellulose, starch, sugar) on rumen fermentation (McDonald et al., 2011).

Murphy et al. (1982) included substrate sources when modeling the impacts of roughage and concentrate-based diets on stoichiometric coefficients of VFA. Soluble carbohydrates (sugars, fructans, beta-glucans (NRC, 2001)) and cellulose produced the greatest stoichiometric coefficients of acetate (Murphy et al., 1982). Propionate stoichiometric coefficients were greatest when protein was the substrate, and molar proportions of propionate doubled with starch from concentrates (Murphy et al., 1982). According to Murphy et al. (1982), if one were trying to maximize propionate supplies, then a high protein, high starch concentrate feedstuff would be best.

Methods to Measure VFA Concentrations

The use of isotopes to understand metabolic changes in various species has been available since the 1930s (Wilkinson, 2018). Isotope studies function by infusing a known amount of a labeled isotope (e.g., C^{13} or C^{14}) and quantifying VFA concentrations. In early isotope-based VFA quantification work, C^{14}, a radioactive isotope, was commonly used. Due to concerns surrounding radioactivity exposure of animals and humans, stable isotope, C^{13}, usage began. There are two ways to deliver a stable isotope infusion: bolus, continuous, or a bolus priming dose in conjunction with continuous infusion (Wilkinson, 2018). Regardless of delivery method, animals must be sampled frequently. Normally, a goal of an isotope infusion is to maintain a metabolic steady state because variation in metabolism could render a tracer undetectable (Wilkinson, 2018). However, a metabolic steady state may not simulate true biological conditions because animals do not constantly consume and would naturally have fluctuations in metabolites.

Isotope-based approaches have been used in previous studies (e.g. Sutton et al., 2003; Storm and Kristensen, 2010) to quantify VFA dynamics to gain more accurate VFA data. However, isotope-based studies are expensive due to isotope costs and human labor. Modelling provides an alternative to isotope-based approaches and is able to provide preliminary predictions or improvements to previous work. An example of a model focusing on VFA absorption kinetics is shown by Storm et al. (2012). This model utilized data previously presented by Storm and Kristensen (2010; 2011), which infused *p*-aminohippuric acid into the mesenteric artery, typically used to measure renal blood flow. The activity of the rumen epithelium was measured using deuterium oxide dilution (Storm et al., 2012).The authors found

rumen epithelial blood flow can play a role in the concentration gradient upheld by the rumen epithelium either preventing or allowing absorption of ruminal VFA (Storm et al., 2012).

Calculating VFA Concentrations

The VFA concentrations are typically obtained from comparing the labeled and unlabeled proportion of VFA after a sample is analyzed by gas-chromatography. As described by Hall et al. (2015), VFA supplies are a function of VFA production, absorption, rumen fluid volume, and rumen fluid passage rate. To simply measure VFA concentrations likely does not accurately describe the dynamics of the rumen ecosystem because there are constant inputs and outputs (fluid entry and exit, presence and absence of substrate, etc.). However, VFA concentrations are still widely accepted in the literature, but should only serve as a proxy to actual VFA supplies.

Another factor that contributes to VFA pool size is the interconversions among different VFA. Interconversion occurs when two VFA are not in equilibrium. Interconversion is defined as the transfer of carbon atoms between carbon chains (Kohn and Boston, 2003). Interconversion can only be measured during isotope infusions because labeled carbons appear in the respective VFA, but also other VFA suggesting transfer of carbon atoms (Sejrsen et al., 2006). Interestingly, Seal and Parker (1994) found when C^{14} labeled propionate was infused, the interconversion ratio of acetate to propionate and propionate to acetate was less than the interconversion ratio without an infusion. This suggests the isotope infusion for propionate to acetate interconversion produced three times the amount of the acetate to propionate interconversion, but interconverted less than half of the no infusion treatment (Seal and Parker, 1994; Sejrsen et al., 2006), which could be due to infusions pushing the animal to exceed normal physiological ranges. Work contributing to understanding VFA interconversions has centered on acetate and propionate and acetate and butyrate. Typically, propionate and butyrate will

interconvert into acetate, but there is potential for “opposite interconversions” when acetate converts into propionate and butyrate. Markantonatos et al. (2006) successfully used a steady state stable isotope infusion method to calculate interconversions between major VFA. Markantonatos et al. (2006) found when comparing high concentrate and low concentrate diets there is increased transfer of carbon atoms between acetate and butyrate (38% for high and 28% for low concentrate diets). Some acetate was converted to propionate and valerate, but conversion of propionate to acetate or butyrate or butyrate conversion to acetate was almost non-existent. Markantonatos et al. (2006) provides insight into a working four pool interconversion model that needs to undergo further testing in order to better understand the nature of potential interconversions. Additional work that has been conducted to describe interconversions between straight-chain VFA and branched-chain VFA is modeling work conducted by Ghimire et al. (2017). Lack of data caused the model to have large prediction errors, therefore, presenting the need for more ruminal VFA interconversion data from animal studies.

The ability and possibility for carbon atom transfer is diet and rumen pH dependent. This ideology stems back to Murphy et al. (1982) discussing the impacts of substrate source and type of feed on stoichiometric coefficients of VFA. Additionally, ruminal pH is dependent upon VFA supplies and lactic acid production and diet, particularly the presence or absence of rapidly fermentable carbohydrates and long-stem forage (Nocek, 1997; Owens et al., 1998). pH depression is also a function of the buffering capacity of the rumen (Dijkstra et al., 2012). Additionally during pH depression, microbial populations are altered and may degrade substrates at different rates causing improper, poor, and inefficient degradation of feed, and ultimately inefficient nutrient absorption (Mould et al., 1983). All of these factors can cause decreased

rumen fluid passage rate and an accumulation of VFA potentially pushing VFA out of equilibrium.

VFA Absorption

Ruminal VFA absorption accounts for 65 to 85% of total nutrients absorbed (Rémond et al., 1995). Volatile fatty acids have two main routes of absorption: passive diffusion (Dijkstra, 1994) and transporter-mediated (Laarman et al., 2016). The relative proportion for a VFA to passively diffuse or use a transporter-mediated channel is variable, and is based on the type of VFA being absorbed. For example, Penner et al. (2009a) and Schurmann (2013) discussed acetate was more likely to passively diffuse (30 to 60%) or use a bicarbonate-dependent transport (42 to 57%). Similarly, butyrate is expected to passively diffuse (25 to 50%) or use bicarbonate-dependent transport (25 to 75%), but has a moderately low tendency to passively diffuse than acetate (Penner et al., 2009a).

There has been conflicting information regarding the form (associated or dissociated) of VFA which are absorbed. Gäbel and Martens (1991) proposed that dissociated VFA, an ionized organic acid (VFA^-), was more likely to passively diffuse (Dijkstra, 1994). Bilk et al. (2005), Aschenbach et al. (2009), and Penner et al. (2009b) found similar responses to Gäbel and Martens (1991) and Dijkstra (1994). Ash and Dobson (1963), Allen (1997) and Laarman et al. (2016), suggested that at a stable rumen pH (pH > 6), VFA passively diffuse in the associated form. Under either paradigm, pH depression would result in greater proportions of dissociated VFA and ultimately impair absorption rate (Allen, 1997).

Gäbel and Martens (1991) also provided a basic understanding to the complexity of the rumen epithelial transporters and their role in VFA and electrolyte movement and pH homeostasis. Rumen epithelial transporters are known to adapt as VFA concentrations change

(Aschenbach et al. 2009; Penner et al., 2009a). For example, Laarman et al. (2013) fed a diet lacking in fibrous feedstuffs with the goal to provoke ruminal VFA supplies to understand rumen epithelial adaptations. Del Bianco Benedeti et al. (2016) fed concentrate diets differing in processing method with the same goal as Laarman et al. (2013). Each study had different types of VFA, but were able to see directional changes of different rumen epithelial transporters. Del Bianco Benedeti et al. (2016) saw decreased expression of sodium hydrogen exchanger isoform 1 (**NHE1**) and sodium hydrogen exchanger isoform 2 (**NHE2**), and increased supply of ruminal valerate. Laarman et al. (2013) saw a higher abundance of monocarboxylate transporter 1 (**MCT1**), a short chain fatty acid transporter, and lower abundance of sodium-bicarbonate cotransporter isoform 1 (**NBC1**) in response to butyrate treatment. Valerate is a BCVFA accounting for < 5% of total VFA, and butyrate is a short-chain fatty acid accounting for approximately 10% of the total VFA produced (Rémond et al., 1995).

One of the major concerns surrounding measuring VFA absorption rate is an accurate and representative method. Absorption rate is a function of rumen fluid volume, fluid entry and exit, rumen papillae absorptive capacity regulated by expression of genes, and ruminal pH. Many studies like Laarman et al. (2013) and Del Bianco Benedeti et al. (2016) only focus on rumen epithelial adaptations, and fail to consider other rumen environment factors that drive VFA and electrolyte absorption. To gain an accurate understanding of VFA absorption, rumen fluid volume and passage rate must be measured along with rumen epithelial gene expression.

Papillae Structure and Function

Rumen papillae, extremely small finger-like projections lining the rumen wall, are essential for ruminal nutrient absorption (Dobson et al., 1956). Volatile fatty acids (Penner et al., 2009a), water, nitrogen in the form of urea or ammonia (Houpt and Houpt, 1968), and different

electrolytes are the main nutrients absorbed from the rumen (Gäbel, et al., 1987; Penner et al., 2009a). The length and width of a papilla is a good external indicator of the absorptive capacity (Gäbel et al., 1987). Papillae with larger surface area are thought to have greater absorptive capacity in comparison to papillae with smaller surface areas. The growth of papillae are stimulated by the presence of VFA, particularly propionate and butyrate (Govil et al., 2017), linking back to the particular diet the animal is fed or when solid feed is introduced to stimulate microbe growth (Lane et al., 2000, Steele et al., 2015). For example, in calves, starter is fed because the grain mixture contains rapidly fermentable carbohydrates which are easier for an immature rumen to digest in comparison to a forage-based diet (Govil et al., 2017). Higher VFA supply results in larger, longer papillae with a greater presence of genes regulating nutrient absorption (Lane et al., 2000). Additionally, Wang et al., (2017) discussed the difference between forage quality and particle size on papillae growth. They found when lactating animals were fed low quality forage, larger particle size increased papillae width (Wang et al., 2017). With this knowledge, the next step could be to provide high quality forage with high nutritional value and larger particle size to investigate papillae width and length responses.

The rumen is described as a non-glandular stomach, which defines the type of epithelium present (Elhamd and Elbab, 2017). The papillae are also referred to as the tunica mucosa. The anatomy of a papilla begins with the keratin layer as the apical-most portion. Papilla keratin is used as an epithelium protective agent. Directly underneath the keratin are the different rumen epithelium strata. These strata, arranged apically to basolaterally, are called corneum, granulosum, spinosum, and basale. The strata corneum, granulosum, and spinosum all have squamous cells (Baldwin and Conner, 2017). The stratum corneum is the most keratinized layer and being the apical-most layer, has the most defined cell wall (Steven and Marshall, 1969). The

keratinization of the corneum provides protection against microbes and changes in rumen pH (Baldwin, 1998). Mitochondria have not been found in the stratum corneum (Kuhn and Thompson, 1965). Because of the lack of mitochondria, the stratum corneum is thought to have the least amount of metabolic activity (Baldwin, 1998). The stratum granulosum consists primarily of granular cells (Dobson, 1956) and there appears to be no clear division between the granulosum and spinosum as there is with other strata (Baldwin, 1998). The stratum granulosum contains majority of the rumen epithelial transporters (Laarman et al., 2013). According to Dobson (1956), the stratum spinosum appears to have intracellular structures resembling human skin "goosebumps" and a complex of fibrils creating "bridges" across the stratum. Later work suggested the structures described by Dobson (1956) are most likely mitochondria and various organelles because the spinosum and basale have the most metabolic activity out of the strata (Baldwin, 1998). The stratum basale consists of well-defined columnar cells, shown to have the greatest mitochondrial density, and are less intensified when immunostaining for Claudin-1 meaning the integrity of the rumen epithelium at the apical-most portion is stronger (Graham and Simmons, 2005). The basement membrane defines the separation between the epithelium and lamina propia. The next layer is the tunica submucosa, a vascularized submucosal layer, will drain the nutrients transported to the lamina propia. Immediately following the tunica submucosa, the tunica muscularis and tunica serosa are the basolateral-most layers.

Rumen Epithelium

The primary roles of the rumen epithelium are to maintain ion homeostasis, serve as a microbial barrier, and absorb VFA. The first route of SCFA transport is associated passive diffusion, when a proton is linked to the fatty acid carbon chain. The second route is dissociated transporter mediated, when negatively charged SCFA utilize a monocarboxylate transporter

(MCT). Monocarboxylate transporters are symporters because a proton is transported with the SCFA to alleviate some of the potential ion gradient disturbances. The rumen epithelium is comprised of multiple layers of stratified squamous epithelium. Each strata, except the stratum corneum, contains different rumen epithelial transporters, which facilitate the movement of short chain fatty acids (SCFA), protons, sodium and bicarbonate ions to maintain rumen pH and the ion gradient. Additionally, each strata contains metabolic genes, which help maintain rumen epithelium integrity (Zhang et al., 2018), consistent protein synthesis (Mayer and Bukau, 2005), and regulate downstream responses like glucose and insulin homeostasis (Hay, 2011).

The location and abundance of the transporters and other metabolic genes is extremely important in understanding the potential adaptations that can occur due to diet changes. Previous work from a colleague's lab, localized genes and found high expression in the stratum granulosum, stratum basale, or throughout the strata. Additionally, transporters that work in tandem with one another are more likely to simultaneously adapt (Yohe, unpublished data).

Rumen Epithelial Transporters

Rumen epithelial transporters are responsible for the movement of SCFA, protons, sodium, and bicarbonate. The general classification of transporter isoforms are monocarboxylate transporters (MCT), sodium-hydrogen exchangers (NHE), anion exchangers (AE), and sodium bicarbonate cotransporters (NBC). These transporters are also present in the omasum, abomasum, small intestine and large intestine, but in lesser abundance than the rumen (Kirat et al., 2006) because majority of absorption occurs in the rumen.

Monocarboxylate Transporters

Monocarboxylate transporters are symporters responsible for the movement of $SCFA^-/H^+$ basolaterally. There are a number of isoforms, but the particular ones participating in VFA transport are MCT1, MCT2, and MCT4. MCT1 is present in the stratum spinosum (Kirat et al., 2006) and the basement membrane of the stratum basale, and typically works in tandem with MCT4 or MCT2 to transport VFA (Graham et al., 2007). MCT2 is present intracellularly in the stratum granulosum and spinosum, but is present on the basement membrane of stratum basale (Graham et al., 2007). MCT4 is also present in the stratum granulosum, spinosum, and basale, but is on the apical membrane of the stratum basale (Graham et al., 2007). MCT2 and MCT4 have approximately the same abundance, but due to MCT2 being slightly more intracellularly in comparison to MCT4, we tend to see changes in transporters more proximal to the lumen or basement membrane (Laarman et al., 2013). Because H^+ accompanies SCFA from the rumen, all MCT isoforms are thought to play a role in ruminal pH regulation (Steele et al., 2013).

Monocarboxylate transporter abundance is a function of diet, which drives rumen conditions. For example, Steele et al. (2011) found that during pH depression the strata decrease in size, but transporter abundance was not measured during this study. Steele et al. (2012) conducted a similar experiment, but investigated genes regulating SCFA transport and ketogenesis. Steele et al. (2012) found that MCT genes did not change in response to a diet shift (from high forage to high concentrate) or an increase in ruminal butyrate concentration. The lack of change in MCT, despite butyrate concentration increases, could be due to measured pH depression. During pH depression, the rumen epithelium appears and functions in a compromised manner. The cohort of animals from this study that did not display pH depression also did not have greater VFA supplies likely not allowing an environment to promote

transporter adaptation. Contrastingly, Laarman et al. (2013) found that MCT1 abundance increased when butyrate was ruminally bolused daily, but did not produce a different ruminal VFA profile in comparison to cows not dosed with butyrate. Both cohort of animals (butyrate infusion versus control) were in an acidotic state potentially causing the epithelium to be compromised as Steele et al. (2012) recognized. Laarman et al. (2013) shows that MCT abundance is dependent upon VFA profile, but ruminal pH depression seems to override VFA influence on MCT.

Sodium Hydrogen Exchangers

Both NHE1 and NHE3 are responsible for recycling protons back to the lumen and importing sodium (Laarman et al., 2013, 2016; Orlowski and Grinstein, 2004). NHE1 and NHE3 are present in the stratum granulosum along the apical membrane (Graham et al., 2007). The exception to this would be NHE2, which is present intracellularly in the granulosum, spinosum, and basale layers (Graham et al., 2007). NHE2 does not control electrolyte movement apically to basolaterally like the other sodium hydrogen exchangers (Graham et al., 2007), but actually controls intracellular sodium concentrations (Orlowski and Grinstein, 2004) NHE2 metabolizes sodium and produces protons which maintains the intracellular charge (Orlowski and Grinstein, 2004). NHE1 and NHE3 isoforms play a significant role in ruminal pH homeostasis (Orlowski and Grinstein, 2004). Changes in sodium hydrogen exchanger isoforms due to ruminal pH depression is demonstrated by Yang et al. (2012) in goats. Yang et al. (2012) found that an increase in dietary concentrates increased VFA concentrations and expression of NHE1 and NHE3. Lu et al. (2016) found similar NHE1 and different NHE2 and NHE3 (decreased) responses to Yang et al. (2012), but Lu et al. (2016) investigated omasal NHE responses instead of ruminal. The likelihood for VFA to be absorbed across the ruminal epithelium is higher than

the omasal epithelium most likely causing the observed differences seen with NHE even though they are not SCFA transporters.

Rumen Epithelium Integrity

Destruction of the rumen epithelium would greatly impair absorptive and metabolic capacity due to projected sloughing and overall epithelium loss. Epithelium disruption normally is caused by an adverse event such as acidosis (Khafipour et al., 2009) or heat stress (Baumgard and Rhoads, 2013). During these conditions, it has been found that gram-negative bacteria concentrations decrease, which normally regulate lipopolysaccharide (**LPS**) a ruminal inflammatory marker (Khafipour et al., 2011). Due to increased concentrations of LPS, an inducer of inflammation, rumenitis can occur ultimately causing further destruction of the rumen environment (Zhao et al., 2018).

Claudin-1 (**CLDN1**) and Gap Junction alpha-1 (**GJA1**) are genes responsible for rumen epithelium integrity maintenance. Claudin-1 has been expressed in the stratum granulosum and stratum basale with some appearance in the stratum spinosum. Claudin-1 gene codes for a Claudin-1, a tight junction membrane protein. Gap Junction alpha-1 can be classified as a connexin, a group of gap junction proteins. Gap Junction alpha-1 codes for a transmembrane protein (Connexin-43) that has a role in gap junction formation and pH regulation. Gap junction alpha-1 is most highly expressed in the stratum granulosum and is less abundant in the spinosum and basale, which is logical because gap junctions need to be in the apical-most portion of the epithelium. Interestingly, during a high concentrate diet, fed to decrease rumen pH, and sodium butyrate treatment, Claudin-1 and GJA1 were both upregulated. It appeared as though the sodium butyrate treatment mitigated the effects of ruminal pH depression and Claudin-1 and GJA1 were able to maintain rumen epithelial integrity (Zhang et al., 2018).

Metabolic Genes

The rumen epithelium metabolic genes selected for investigation have a direct role in SCFA metabolism, mTOR signaling, glucose and insulin homeostasis, or protein folding. There are four enzymes involved in the pathway converting Acetyl-CoA molecules into beta-hydroxybutyrate (**BHB**): acetoacetyl-CoA synthase (**AACS**), 3-hydroxy-3-methylglutaryl- CoA synthase 12 (**HMGCS2**), 3-hydroxymethyl-3- methylglutaryl-CoA lyase (**HMGCL**), 3-hydroxybutyrate dehydrogenase, type 1 (**BDH1**). The production of BHB is called ketogenesis because BHB is a ketone body. Thus, the genes regulating this process are called ketogenic genes. Additionally, AACS, HMGCS2, and HMGCL regulate enzymes involved in the melanovate pathway, which produces isopentenyl pyrophosphate the precursor to all isoprenoids. Despite the importance of isoprenoids, we will remain focused on how these genes regulate acetate and butyrate metabolism and BHB production.

Acetoacetyl-CoA Synthase

Acetoacetyl-CoA synthase (**AACS**) is involved in acetate metabolism (Okamura et al., 2010). Acetoacetyl-CoA synthase is part of the thiolase family, which regulate the conversion of two acetyl-CoA molecules to acetoacetyl-CoA. Acetoacetyl-CoA synthase behaves similarly to a thiolase, but helps convert acetoacetate (acetyl CoA and malonyl CoA) to acetoacetyl-CoA rather than the conversion of 2 acetyl-CoA molecules. Naeem et al. (2012) found no significant change in AACS expression during high protein calf starter, which could be due to the calf having an immature rumen or the lack of BHB concentration changes.

3-Hydroxy-3-Methylglutaryl-CoA Synthase 2

3-Hydroxy-3-Methylglutaryl-CoA Synthase 2 (**HMGCS2**) is responsible for the conversion of acetoacetyl-CoA to B-hydroxy-B-methylglutaryl-CoA (HMG-CoA). Naeem et al., (2012) found HMGCS2 to be the most abundant metabolic gene of the rumen epithelium in pre-weaned calves. Naeem et al., (2012) believed HMGCS2 was upregulated as a coping mechanism to make sure enough substrate (HMG-CoA) was available for BHB synthesis. Leighton et al. (1983) reported similar results to Naeem et al., (2012) in mature sheep. However, it is possible the mature bovine rumen epithelium will respond differently because of diet differences between the two species, allocation of energy (meat vs wool vs milk production), and rumen development management during pre-weaning.

3-hydroxy-3-methylglutaryl-CoA lyase

A lyase will catalyze a reaction by the formation of new double bond without hydrolysis. 3-hydroxy-3-methylglutaryl-CoA lyase (**HMGCL**) helps remove Acetyl-CoA from HMG-CoA and forms a double bond with an oxygen to form acetoacetate (Naeem et al., 2012). Naeem et al. (2012) found that when calves were fed a high protein starter in comparison to a control starter there was an upregulation of HMGCL despite blood BHB remaining unchanged.

3-hydroxybutyrate dehydrogenase type 1

3-hydroxybutyrate dehydrogenase type 1 (**BDH1)**, is the last gene regulating production of BHB. BDH1 converts acetoacetate into BHB**.** Acetone is also produced from the breakdown of acetoacetate into BHB, but that reaction is catalyzed by a different enzyme. Similar to HMGCL results in Naeem et al. (2012), despite blood BHB concentrations being unaffected by treatment, BDH1 was upregulated when a high protein starter was fed. Work by Naeem et al.

(2012) suggests that ketogenic genes present in the rumen epithelium will respond to high protein dietary concentrations in calves, but this response may not be indicative of mature bovine epithelium because the calves in Naeem et al. (2012) had an underdeveloped, immature rumen.

Serine-Threonine Protein Kinase

Serine-threonine protein kinase or protein kinase B (**AKT1**), is responsible for a number of things, but plays a main role in mTOR signaling allowing for increased cell growth and differentiation, and increased protein synthesis (Dienstmann et al., 2014). In both mice and fruit flies, AKT1 was found to play a main role in insulin homeostasis. Protein kinase B isoforms are activated in a similar manner to mTOR signaling through Phosphatidylinositol-4,5-bisphosphate 3-kinase (**PI3K**) and Phosphatidylinositol (3,4,5)-trisphosphate **(PIP3**). Phosphatidylinositol-4,5-bisphosphate 3-kinase and PIP3 are activated by insulin or insulin-like growth factor (IGF-1) (Hay, 2011). Downstream effects of AKT1 are isoforms of forkhead transcription factors (FOXOs; Hay, 2011). When activated, FOXOs will inhibit pancreatic B-cell causing insulin to not respond appropriately to changes in blood glucose (Hay, 2011). AKT1 inhibits FOXOs, so insulin can respond appropriately.

Heat Shock Protein 70

The heat shock protein (HSP) family is classified as a protein chaperone. A chaperone is responsible for the correct folding and unfolding of proteins. HSP function is particularly apparent when newly synthesized proteins are starting to denature due to a stressed situation. HSP70, specifically, is the most understood HSP (Mayer and Bukau, 2005). It has three primary roles: to prevent aggregation of newly synthesized proteins, fixing aggregated proteins, and completing folding of proteins (Mayer and Bukau, 2005). HSP70 is important to investigate in

the rumen epithelium because if diets cause downregulation of HSP70, then protein function could be impaired.

Propionate Metabolism Genes

One thing to recognize about the previously discussed genes is none are involved in propionate metabolism. This is logical because majority of propionate metabolism occurs in the liver (Aschenbach et al., 2010), so genes regulating propionate metabolism would not be present in the rumen epithelium. If any gene would relate to propionate, it would be the SCFA transporters rather than a metabolic gene.

Background on Post-Absorptive System

The post-absorptive system can be described in two-fold. The first level is the digestive tract venous complex that shuttles absorbed nutrients from the reticulo-rumen, omasum, abomasum, small intestine, and large intestine to the liver, various muscles, peripheral tissues and kidneys. The second level is the actual metabolism of nutrients at their respective locations. Absorbed nutrients are viewed as substrates to different metabolic processes. For example, propionate, absorbed from the rumen, is catabolized in the liver to produce glucose. Glucose is a substrate for microbial fermentation in the rumen preempting liver gluconeogenesis (Zhang et al., 2016).

Impact of Starch on Post-Absorptive System

The impact of starch on the post-absorptive system will occur indirectly through blood metabolite concentration changes. Extending into the periphery, the blood metabolite profiles are most likely going to change due to hepatic gluconeogenesis and overall liver metabolism (Zhang et al., 2016). The type of substrate provided to liver metabolism also drives the relative losses in

the system. For example, lactate produces hepatic glucose with more loss (lower efficiency) than propionate, which has very little carbon loss (Armentano, 1992).

Impact of Fiber on Post-Absorptive System

Like starch, fiber does not have a direct impact on the post-absorptive system, but impacts it by producing different VFA. Ruminal fiber digestibility is thought to increase with increased presence of BCVFA, and recent work by Beckett et al. (unpublished data; Chapter 3) found significant connections between fiber source and an increase in BCVFA molar proportions, concentrations, and fluxes. Branched-chain volatile fatty acids also interconvert into branched-chain amino acids (BCAA) (Yang, 2002). Branched-chain amino acids are believed to be helpful for muscle protein synthesis in humans (Wolfe, 2017), and are known to activate the mTOR signaling pathway, increase glucose uptake, and maintain mammary gland health and milk synthesis (Zhang et al., 2017). Both animal experiments and modeling work have centered around optimizing BCAA utilization in the post-absorptive system to decrease losses (Hanigan et al., 1997). Lastly, similar to the rumen, the post-absorptive system has metabolic adaptations during adverse events such as, heat stress (Baumgard and Rhoads, 2013).

Conclusion

Nutrient disappearance has the potential to impact VFA dynamics and the rumen environment directly, and can alter the post-absorptive metabolism indirectly. However, the nature of ruminal and post-absorptive adaptations to changes in nutrient disappearance have not been well-defined. From the work described, accurately quantifying rumen VFA dynamics, rumen epithelial adaptations, and post-absorptive blood metabolite concentrations in response to starch and fiber sources is needed to optimize digestive and feed efficiency.

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Figures

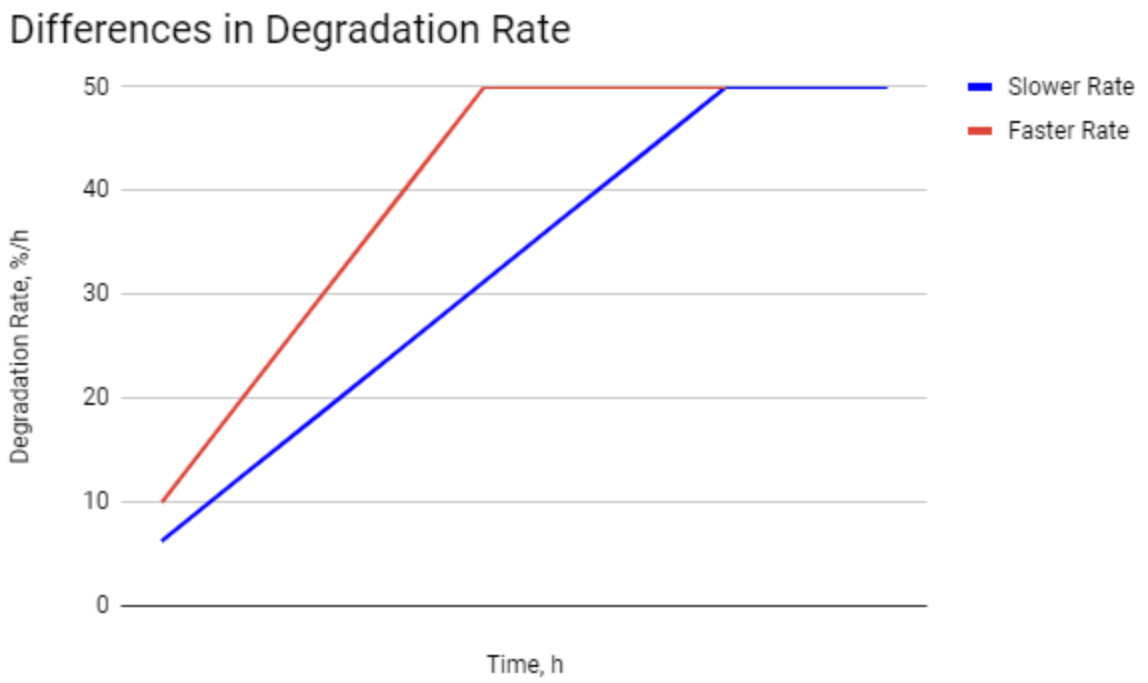

Figure 1.1: Visual description of different ruminal degradation rates. Degradation rate is equivalent to the slope (i.e. rate of change) of a line. Different degradation rates will demonstrate different slopes, but may have the same extent of degradation as exhibited in this figure.

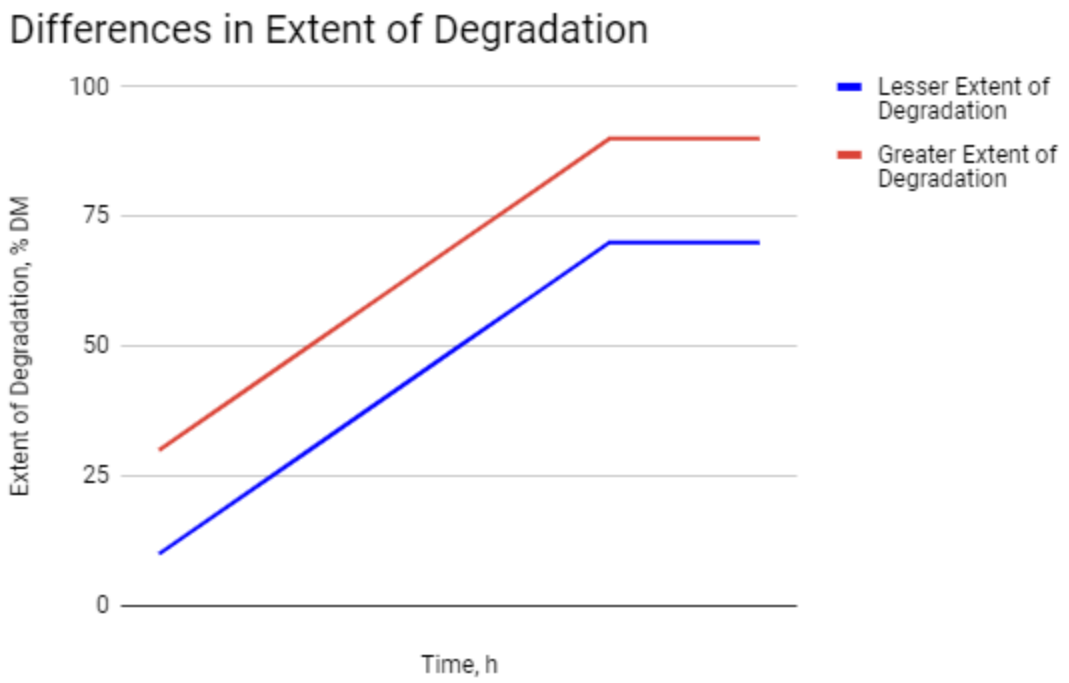

Figure 1.2: Visual representation of different extents of ruminal degradation. Feedstuffs with different extents of degradation, but not different degradation rates, will have the same slope. Different extents of degradation will have different ending "y" values representing different dry matter percentage degraded.

Tables

Table 1.1: Calculated ATP conversion efficiencies for glucose, acetate, propionate, and butyrate as presented by Baldwin (1968).

Metabolite	**Hc[1] (kcal)**	**ATP[2] (kcal)**	**Efficiency[3]**
Glucose	686	304	44%
Acetate	209	80	38%
Propionate	367	144	39%
Butyrate	523	216	41%

[1]Hc: Heat of combustion for each metabolite measured in kilocalories.
[2]ATP: Amount of ATP produced by each metabolite measured in kilocalories.
[3]Efficiency: The ATP conversion efficiency for each metabolite expressed as a percentage. Percentages are calculated by dividing the heat of combustion (kcal) for each metabolite by ATP (kcal).

CHAPTER TWO

Adaptation of a common cell culture assay to rank cattle based on skeletal muscle metabolic activity

This chapter will be published as: Beckett, L., R. Rosemond, B. Renquist, and R.R. White. 2019. Technical note: a muscle biopsy technique for stratifying cattle by skeletal muscle metabolic activity. Journal of Dairy Science.

ABSTRACT

Tissue biopsy metabolic activity, assessed using the oxidation-reduction indicator Resazurin, may serve as a proxy to assess energy expenditure associated with maintenance in non-growing animals or growth rate in growing animals. Herein, we evaluate the repeatability, practicality, and sensitivity of a Resazurin-based assay for ranking bovine skeletal muscle biopsies based on metabolic activity. Six yearling Holstein heifers (**BW** = 330 ± 11.3 kg) were fed 4 dietary treatments consisting of high or low rumen degradable starch and fiber arranged factorially in a partially replicated Latin Square design. Periods were 18 d long, consisting of 3 d diet transition, 14 d diet adaptation, and 1 d sample collection. Semitendinosus biopsies were collected into ice cold Dulbecco's modified eagle media (DMEM) from each heifer during each period. Analysis was initiated within an hour of sample collection. To assess tissue metabolic rate, biopsies were transferred to DMEM with Resazurin and incubated at 37^0C. Fluorescence of each sample was read at time 0 and at 15-minute intervals for two hours. Change in fluorescence was representative of skeletal muscle reducing equivalent production. Fluorescent signal strength increased with time ($P < 0.001$) and relative rank of treatments did not change with time ($P > 0.05$). Accordingly, future studies may compare fluorescence at a single time point. Change in fluorescence at 120 minutes was used for analysis of the fixed effects of fiber, starch, and animal, when accounting for a random effect of period. Samples collected when animals were on a high ruminally degradable starch diet were more metabolically active than samples collected from animals on low starch diets ($P = 0.023$). Significant differences in metabolic activity among individual animals were also identified. Average relative fluorescence was correlated with dry matter intake (**DMI**), average daily gain (**ADG**), and feed to gain ratio (**F:G**). The relative fluorescence tended to correlate with ADG (r = 0.749; $P = 0.0864$) and F:G (r = -0.783; $P = 0.066$). Change in fluorescence did not

correlate with DMI (P = 0.773). Although evaluated on a small sample size, this technique shows promise as a potential means of ranking animals by growth or feed efficiency. Further work on a larger experimental population is needed to confirm the usefulness of this assay as a consistent and reliable predictor of these important phenotypic parameters.

Keywords: bovine, skeletal muscle, metabolic activity

Basal Metabolic Rate Measurements

Selecting feed efficient animals will help dilute maintenance energy requirements and enhance feed efficiency, improving dairy cattle production system sustainability (White, 2016). One way to select animals with improved feed efficiency is to identify those with particularly efficient metabolism. The energy requirements of cattle are related to maintenance, growth, reproduction, and lactation. Maintenance energy expenditure and efficiency of energy use can be measured by either using metabolic chambers that collect heat and gas produced (Ellis, et al., 2006) or performing large serial slaughter studies (Lofgreen and Garrett, 1968). However, the practicality of these approaches has declined over time due to the cost of conducting studies using metabolic chambers and challenges with justifying terminal use of large numbers of animals. As a result, our knowledge of how to manipulate energy use efficiency is primarily from cattle with older genetics, and older feeding strategies (e.g., Lofgreen and Garrett, 1968). Recent work on the genetic bias of feed efficiency suggests there is considerable between-animal variation in efficiency of energy use (Hardie, et al., 2017). In turn, a high-throughput method for assessing energy expenditure may allow for selection to improve efficiency.

A technique recently developed for measuring metabolic activity in embryonic zebrafish (Renquist et al., 2013; Williams and Renquist, 2016) and skeletal muscle biopsies from tilapia

(Kentch et al., 2017) may be applied to assess metabolic activity in cattle, which could serve as one such phenotyping approach. In brief, this assay applies Resazurin, an oxidation-reduction indicator, which is highly fluorescent upon becoming irreversibly reduced by NADH, FADH2, or cytochrome C. This technique has not previously been tested in cattle. We hypothesized that by applying this technique in cattle skeletal muscle biopsies we could assess the change in metabolic activity induced by changes in the diet or inherent animal variation in feed efficiency and segregate individuals based on metabolic activity.

The objectives of this study were to: 1) investigate the application and sensitivity of this novel fluorescent assay using muscle tissue biopsies collected from Holstein heifers; 2) evaluate the application of this test to quantify the metabolic response to dietary changes; and 3) apply the test to identify inherent individual animal variation in metabolic activity.

Treatment Diets and Adaptation Period

Six ruminally cannulated yearling (11 mo ± 7 d) Holstein heifers (**BW** = 330 ± 11.3 kg) were randomly assigned to 4 dietary treatments in a partially replicated Latin Square design. The square was partially replicated because there were 6 animals and 4 treatments meaning that two treatments were duplicated in each period and the other two treatments were not. Each period change corresponds to a change in treatment diet. Periods were 18 d in length. The first 3 d were used to adapt animals between rations, animals consumed the treatment ration for 14 d, and samples were collected on the final day. Diets were factorially designed with corn silage as a base (29.4 to 35.6, % DM), and used different dietary inclusion combinations of ground barley (high rumen degradable starch [HS] 14.1 to 14.8 % DM; low rumen degradable starch [LS] 0.310 to 0.670, % DM) or ground corn (HS 0.00 to 0.380 % DM; LS 10.5 to 12.3 % DM) and pelleted beet pulp (high rumen degradable fiber [HF] 3.55 to 6.05 % DM; low rumen degradable fiber [LF] 30.2

to 32.9 % DM) or timothy hay (HF 19.9 to 25.0 % DM; LF 0.020 to 1.43, % DM) to generate rations with high and low ruminal degradable starch and fiber. As described in Table 1, diets also contained soybean meal (HS-HF 9.3 % DM; HS-LF 17.1 % DM; LS-HF 9.95 % DM; LS-LF 15.3 % DM), blood meal (HS-HF 3.78 % DM; HS-LF 0.00 % DM; LS-HF 4.37 % DM; LS-LF 0.040 % DM), and corn gluten feed (HS-HF 3.26 % DM; HS-LF 0.00 % DM; LS-HF 1.65 % DM; LS-LF 7.22 % DM) to make them isonitrogenous. These treatment combinations resulted in divergent energy supply to the animals (NRC, 2001), and the calculated metabolic energy (**ME**) for each ration is included in Table 1.

Sample Preparation and Collection

A 10 cm wide area 5 cm to 35 cm ventral to the point of the ischium was shaved and scrubbed three times with betadine and isopropanol. Ten ml of lidocaine was administered subcutaneously in 5 to 6 locations radially arrayed 2 cm externally to the biopsy site. The target biopsy site was 20 cm ventral to the point of the ischium. Muscle tissue samples were collected by making a 1 cm incision through the skin with a #20 scalpel blade, inserting a 20 gauge biopsy needle (Bard® Mission® Disposable Core Biopsy Instrument, Bard Biopsy, Tempe, AZ, USA) to a 4 cm depth, and depressing the needle collection sheath to obtain a sample. Three biopsies, with the aim of obtaining approximately 30 mg of sample, were collected per animal per period to evaluate how variation in sample collection (sample mass, collection site within the muscle, and other unknown factors) influenced consistency of the results. Samples were not weighed after collection because an analytical balance was not available at the farm. The incision site was sealed using monofilament #2 suture wire, cleaned with isopropanol, and sprayed with adhesive bandage. The right semitendinosus was sampled in periods 1 and 3 and the left semitendinosus was sampled in periods 2 and 4 allowing muscle to properly regenerate and animals to heal.

Immediately following collection, the biopsies were placed in individual wells of a 96 well plate filled with a pre-test solution. The pre-test solution was formulated as described in Renquist et al. (2013) and contained 30 ml DMEM (Fisher Scientific 21-041-025, Waltham, MA, USA), 7.5 mg Fungizone (Fisher Scientific 15-290-026), 0.12 mg Chloramphenicol (Fisher Scientific BP904-100), and 0.03 mg Ampicillin (Fisher Scientific AAJ6097714). After all samples were collected, they were transferred from the pre-test solution into individual wells of a 96 well plate filled with AlamarBlue (Thermo Scientific Y00-100) Test Assay solution. The test solution was identical to the pre-test solution with 1.6% AlamarBlue added. Solutions were mixed immediately prior to biopsy collection, filtered using a sterile 0.22 µM filter, and warmed to 37^0C before use.

Sample Analysis

Directly after sample collection, the live muscle tissue samples were transported to the lab in the pre-test solution on ice, transferred to the test solution, and analyzed. Analysis was initiated within one hour of tissue collection. When samples were transferred to the test solution, the test solution plate was incubated in the plate reader (Spectramax M5; Molecular Devices, LLC, San Jose, CA) at 37℃. Fluorescence was read at time 0 and every 15 minutes for 2 hours using excitation and emission wavelengths of 530 and 590 nm, respectively (Renquist, et. al, 2013; Williams and Renquist, 2016). Soft Max Pro 6.1 (Molecular Devices, LLC, San Jose, CA) was used to quantify resulting emissions, and relative fluorescence (standardized to time 0) was calculated at each time point for each sample.

Consistency of Samples within Animal-Periods

Triplicate samples were collected within an animal-period to evaluate the consistency of animal rankings obtained from like samples. The within-animal-period CV for relative

fluorescence was approximately 20%, suggesting a need to standardize samples by protein or DNA content, as was done by Renquist et al. (2013), or by mass. Despite the degree of variation among samples collected from the same animal consuming the same diet, a Wilcoxon rank sum test did not find significant differences in the rankings of animals when different individual samples were used ($P>0.05$) suggesting this variation did not impede opportunities to rank animals by skeletal muscle metabolic activity.

Statistical Analysis

Mean relative fluorescence was calculated for each animal-period at each time point read. Statistical analysis of these mean fluorescence data was conducted in R version 3.1.0. (R Core Team, 2014). Analysis was structured into 2 questions: 1) does the incubation time change the perceived influence of a treatment on relative NAD^+ reduction rate; and 2) are differences between animals and treatments discernable after 2 h. To address the first question, a linear mixed effect model was used to test how time, fiber and starch digestibility, animal, and period influenced standardized fluorescence readings. Fiber, starch and time were fixed effects, and the 2- and 3-way interactions among these fixed effects were also evaluated. Period and animal were treated as random effects. A significant time by starch or fiber interaction would suggest that treatment differences were not consistent across a sampling period and some ideal fluorescence reading time would need to be determined to make reliable inferences from the data. To address the second question, data from the last time point (2 h) was analyzed using a linear mixed effect model with fixed effects of fiber, starch, and animal and a random effect of period. Significant starch, fiber or animal effects would suggest that the assay (using only a 2 h fluorescence time) was sensitive enough to rank samples based on animal-level factors (genetic potential or diet).

Time

Because the plate can be read at multiple time points, we were able to test the effect of time on within-sample fluorescence (Figure 1). The reduction reaction that causes Resazurin to fluoresce is irreversible, and as a result the signal accumulates with time allowing small short-term differences to amass as time of incubation is extended. The total signal increased linearly with time ($P < 0.0001$). Thus, the tissue biopsies maintained a constant metabolic rate throughout the incubation period. Time did not differentially affect fluorescence based on starch ($P = 0.5106$) or fiber ($P = 0.8072$) in the diet, supporting the conclusion that the rankings of samples were similar if evaluated at any time point within the 2 hours.

Diet

Skeletal muscle biopsies taken when heifers were on a high ruminally degradable starch diet had a higher relative fluorescence than biopsies taken when heifers were fed a diet low in ruminally degradable starch ($P = 0.023$) (Table 2). The link between starch source and skeletal muscle metabolic activity could be caused by a change in energy availability. However, calculated ME in the diets changed more with rumen degradable fiber than with rumen degradable starch (Table 1), making this explanation unlikely. Alternatively, the increase in tissue metabolic activity with increased ruminally degradable starch may be related to a different absorbed volatile fatty acid profile. Different VFA are used for energy with different efficiencies in the post-absorptive system (Baldwin, 1968). Approximate VFA contribution to muscle is dependent upon individual VFA metabolism. Less than 30% of acetate (Knowles et al., 1974), 40 to 55% of propionate (Wiltrout and Satter, 1972), and minimal butyrate is available to the periphery (Bergman and Wolff, 1971). Glucose contribution to skeletal muscle in ruminants is due to gluconeogenesis. Hepatic uptake of propionate, valerate, and isobutyrate allows for increased gluconeogenic

substrate (Aschenbach et al., 2010). If the different starch sources contributed to different profiles of absorbed VFA, it is possible that the absorbed VFA profiles contributed to the effect of ruminally degradable starch on skeletal muscle metabolic activity. Independent of the mechanism driving this dietary effect, our results suggest the assay can be applied to understand the metabolic effects of ration changes.

Animal

Comparisons among animals show that skeletal muscle metabolic activity varied between individuals (P = 0.003). The effect of diet was consistent amongst animals, and animals ranked similarly across diets (Figure 2). This consistency suggests that inherent differences in skeletal muscle metabolic activity may be distinguishable across conditions, making it a possible way to inexpensively and durably screen animals for energy expenditure associated with growth or in non-growing animals, maintenance.

The metabolic activity of a ruminant is a function of body mass and metabolic flux in an inactive, thermoneutral environment (White and Seymour, 2003). These differences in metabolic flux contribute to variability in feed efficiency. Feed efficiency is also influenced by genetics (e.g., Koch et al., 1963), activity and behavior (e.g., Shaver, 2010; Socha et al., 2007), environment (e.g., Socha et al., 2007), and heifer rearing practices (e.g., Khan et al., 2011). The average skeletal muscle metabolic activity (expressed as relative fluorescence) of each individual was paired with dry matter intake (**DMI**), average daily gain (**ADG**), and calculated feed to grain ratio (**F:G**) (Figure 3). Relative fluorescence tended to correlate with ADG (r = 0.749; P = 0.0864) and F:G (r = -0.783; P = 0.066). Change in fluorescence did not correlate with DMI (P = 0.773) (Figure 3). Because of concern with potential high-influence points, a leave-out-one analysis was performed to assess how correlations changed when each point was iteratively removed from the dataset.

Correlations between relative fluorescence and ADG ranged from 0.701 and 0.855 with a mean of 0.778. The F:G correlations ranged from -0.540 and -0.875 with a mean of -0.763. The relationship between DMI and relative fluorescence varied more substantially (range from 0.186 to 0.542, mean 0.187). These results suggest that one animal may have been the primary reason for limited relationship between DMI and relative fluorescence. When this animal was removed, the strength of the F:G relationship also decreased. However, removing data from that animal did not substantially alter the relationship identified between relative fluorescence and ADG. The inconsistency across the dataset suggests the need for additional trials to confirm the relationship between the outputs of this assay and animal productivity.

Skeletal muscle biopsy metabolic activity was indicative of energy expenditure for growth and maintenance in these young, rapidly growing heifers. The tendency for a consistent positive correlation with growth suggests that variation in the metabolic activity assay measurements correlated with variation in animal growth, as would be expected from the measurement. However, the inconsistency of correlations among the relative fluorescence and DMI or F:G highlights a limitation of the small sample size used in this preliminary assay evaluation. Additional screening on a larger sample size is needed to determine whether this assay could be useful in selecting animals with higher rates of gain or greater feed efficiency.

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Tables

Table 2.1: Ingredients and nutrient composition for each treatment diet expressed on a dry matter basis. Significance is determined by $P < 0.05$ and tendency by $P < 0.10$.

Descriptor	HS-LF[1]	HS-HF[2]	LS-LF[3]	LS-HF[4]
Ingredients, % DM				
Timothy Hay	0.020	19.9	1.43	25.0
Beet Pulp	30.2	6.05	32.9	3.55
Ground Corn	0.000	0.380	12.3	10.5
Ground Barley	14.8	14.1	0.310	0.670
Corn Silage	35.6	34.4	29.4	35.2
Corn Gluten Feed	0.000	3.26	7.22	1.65
Soybean Meal	17.1	9.30	15.3	9.95
Blood Meal	0.000	3.78	0.040	4.37
Vitamin E Premix				
Vitamin A-D3 Premix				
Nutrient, % DM				
DM	73.4	64.8	74.7	63.9
OM	94.8	94.9	94.9	94.7
NDF	35.0	35.0	35.0	35.0
ADF	23.4	23.0	21.1	21.5
Starch	20.0	20.0	20.0	20.0
CP	16.0	15.9	16.0	15.9
Estimated ME, Mcal/kg	2.74	2.63	2.75	2.62
Animal Parameters				
Dry Matter Intake, kg/d	10.6	11.6	10.9	12.3
Average Daily Gain, kg/d	0.90	0.81	0.76	0.60
Feed to Gain Ratio (kg/kg)	11.8	14.3	14.3	20.5

[1]HS-LF = high rumen degradable starch, low fiber treatment

[2]HS-HF = high rumen degradable starch, high fiber treatment

[3]LS-LF = low rumen degradable starch; low fiber treatment

[4]LS-HF = low rumen degradable starch; high fiber treatment

Table 2.2: Relative fluorescence treatment means, standard error, and *P*-values for measuring skeletal muscle metabolic activity in bovines fed different ruminal degradable starch and fiber diets. Significance is determined by $P < 0.05$ and tendency by $P < 0.10$.

Treatment	Relative Fluorescence	SE
HS-LF[1]	0.567	0.038
HS-HF[2]	0.687	0.038
LS-LF[3]	0.537	0.038
LS-HF[4]	0.537	0.038
***P*-values**		
Starch	0.023	
Fiber	0.226	
Starch x Fiber	0.120	
Animal	0.003	

[1]HS-LF = high rumen degradable starch, low fiber treatment

[2]HS-HF = high rumen degradable starch, high fiber treatment

[3]LS-LF = low rumen degradable starch; low fiber treatment

[4]LS-HF = low rumen degradable starch; high fiber treatment

Figures

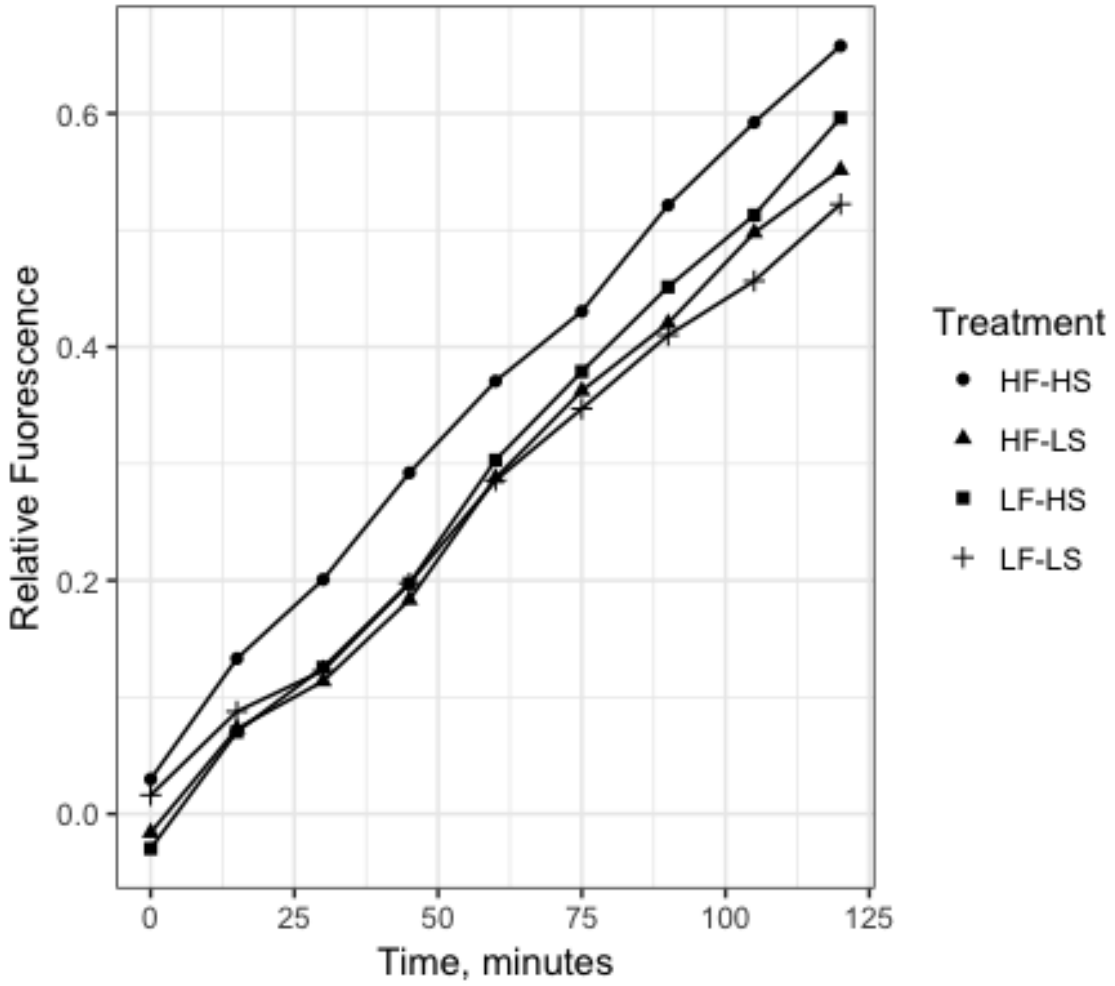

Figure 2.1: Relative fluorescence, equivalent to energy expenditure, analyzed over a 2-hour period for high fiber-high starch (HF-HS), high fiber-low starch (HF-LS), low fiber-high starch (LF-HS), and low fiber-low starch (LF-LS) diets. Values reported are least-square means, the raw values for time point 0 were 0 for all treatments as would be expected from relative fluorescence data. High starch diets had greater relative fluorescence in comparison to low starch diets, but there was no significant effect of fiber or the interaction (Starch $P = 0.023$; Fiber $P = 0.23$; Starch x Fiber $P = 0.12$). The total signal increased linearly with time ($P < 0.0001$), but time did not differentially affect fluorescence (Starch $P = 0.51$; Fiber $P = 0.81$; Starch x Time $P > 0.10$; Fiber x Time $P > 0.10$; Starch x Fiber x Time $P > 0.10$). Significance is determined by $P < 0.05$ and tendency by $P < 0.10$.

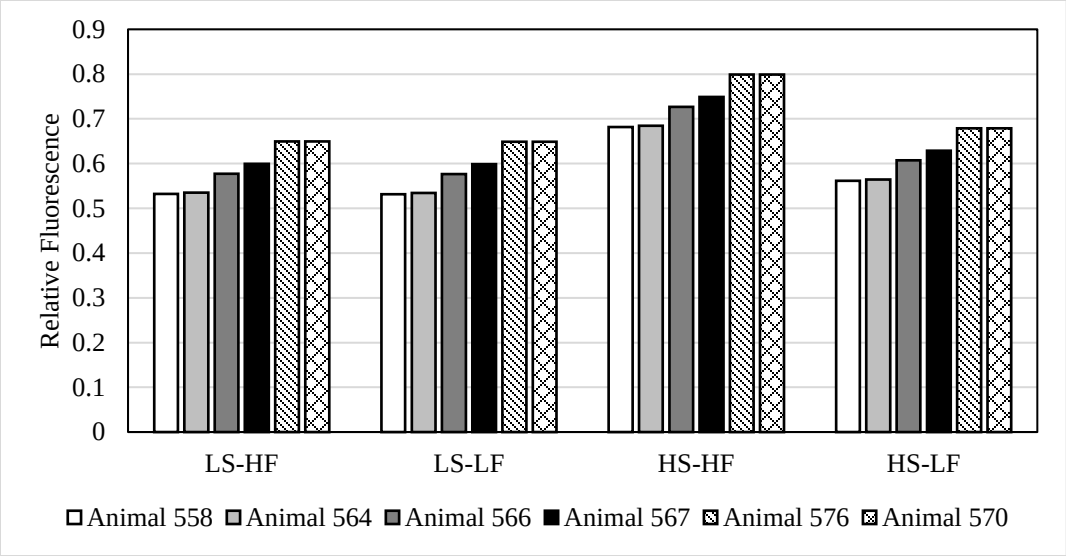

Figure 2.2: Least squares mean relative fluorescence (nm) for each animal, organized by treatment. Treatments included either a high (HS) or low (LS) rumen degradable starch source and either a high (HF) or low (LF) rumen degradable fiber source. Animal numbers are arbitrary and reflect the animal identifier assigned by the farm. These identifiers are included in the legend to show consistency of animal rankings across diets. Comparisons among animals show that skeletal muscle metabolic activity, measured by relative fluorescence of skeletal muscle, varied between individuals ($P = 0.003$). Significance is determined by $P < 0.05$ and tendency by $P < 0.10$.

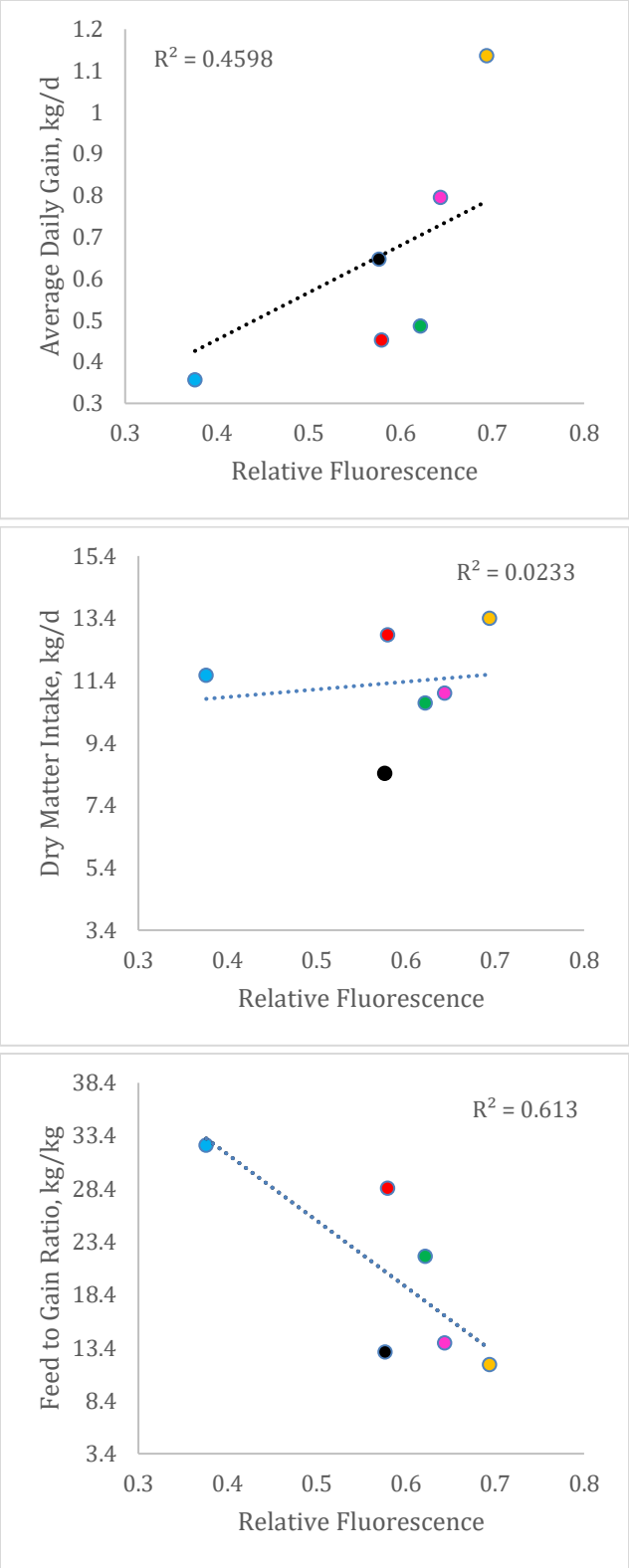

Figure 2.3: Mean relative fluorescence of each animal mapped against corresponding study mean dry matter intake (DMI), average daily gain (ADG), and mean feed to gain (F:G). Data points describe individual animal performance correlated to relative fluorescence. Each color

corresponds to an individual animal. The dotted line shows a best-fit linear regression of the performance variable on relative fluorescence. The R^2 values presented reflect the coefficient of determination for each regression. Relative fluorescence tended to correlate with ADG (P = 0.0864) and feed to gain (P = 0.066). Change in fluorescence did not correlate with DMI (P = 0.773). Significance is determined by $P < 0.05$ and tendency by $P < 0.10$.

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CHAPTER THREE

Rumen volatile fatty acid molar proportions, rumen epithelial gene expression, and blood metabolite concentration responses to ruminally degradable starch and fiber supplies

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Running Head: RUMEN RESPONSES TO DEGRADABLE NUTRIENTS

INTERPRETIVE SUMMARY: Beckett et al. (TBD). "Rumen volatile fatty acid molar proportions, epithelial gene expression, and blood metabolite concentration responses to ruminally degradable starch and fiber supplies" discusses the influence of starch and fiber sources on ruminal volatile fatty acid molar proportions, calculated VFA pool size, and flux. Changes in ruminal volatile fatty acid supplies due to different starch and fiber sources causes the rumen epithelium to mirror VFA fluctuations, but post-absorptive blood metabolites do not convey changes in ruminal VFA supplies.

Rumen volatile fatty acid concentration, rumen epithelial gene expression, and blood metabolite concentration responses to ruminally degradable starch and fiber supplies

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ABSTRACT

The objective of this work is to characterize how rumen VFA concentrations, rumen epithelial gene expression, and blood metabolite concentrations respond to diets containing different starch and fiber sources. A partially replicated Latin Square experiment with four treatments consisting of barley or corn and timothy hay or beet pulp arranged as a 2 x 2 factorial was used to assess shifts in ruminal VFA and fluid dynamics; epithelial gene expression; and blood metabolite concentrations. Beet pulp and timothy hay were used to create relative changes in apparent ruminal fiber degradability; and corn and barley grain were used to create relative changes in apparent ruminal starch degradability. Six ruminally cannulated yearling Holstein heifers (**BW** = 330 ± 11.3 kg) were used. Each period consisted of 3 d of diet adaptation and 15 d of dietary treatment. *In situ* degradation of fiber and starch were estimated from bags incubated in the rumen from d 10 to 14. From d 15 to 17 rumen fluid was collected every h from 0500 to 2300. On d 15 at 0500, a bolus dose of polyethylene glycol was administered and time-series samples for 12 h were used to estimate fluid passage and pool size. Rumen fluid samples were pooled by animal/period and analyzed for pH and VFA concentrations. On d 18, 60 to 80 papillae biopsies were cut from the epithelium and preserved for gene expression analysis. Blood samples were collected from the coccygeal vein for plasma acetate and propionate analysis using gas chromatography mass spectrometry (GCMS) and colorimetric determination of plasma glucose, insulin, and beta-hydroxybutyrate (BHB).

In situ ruminal starch degradation rate (barley 7.61 to 10.5 %/h vs corn 7.30 to 8.72 %/h; $P = 0.05$) and fiber extent of degradation (timothy hay 22.2 to 33.4 % of DM vs beet pulp 34.4 to 38.7 % of DM $P = 0.0007$) were affected by starch and fiber source, respectively. Analysis of molar proportions showed a shift from propionate ($P = 0.06$) to acetate ($P = 0.02$) on timothy hay diets. There was also a shift from valerate ($P = 0.10$) to isovalerate ($P = 0.008$) molar proportions

on timothy hay diets. Comparing molar proportions on corn versus barley diets, there were trade-offs between propionate (P = 0.02) and butyrate (P = 0.04) with corn diets favoring propionate over butyrate. Corn diets also had high molar proportions of valerate (P = 0.049). Neither rumen fluid volume (Starch P = 0.31; Fiber P = 0.75; Starch x Fiber P = 0.55) nor fluid passage rate (Starch P = 0.68; Fiber P = 0.80; Starch x Fiber P = 0.30) were affected by treatment. Expression of 4 genes, heat shock protein 70 (HSP70), beta-hydroxybutyrate dehydrogenase (BDH1), sodium hydrogen exchanger isoform 3 (NHE3) and monocarboxylate 4 (MCT4), were by timothy hay. Acetate was the only blood metabolite affected by diet (Starch x Fiber P = 0.067). Data suggested shifts in VFA molar proportions and epithelial transporters did not necessarily convey shifts in calculated ruminal VFA pool size or blood metabolite concentrations.

Keywords: rumen epithelium, blood metabolites, VFA concentration

Introduction

Volatile fatty acids (VFA) are an innate energy source for ruminants that contribute approximately 70% to 80% to energy requirements (Bergman, 1990). Ration changes cause adaptations within the rumen microbial community which alter fermentation patterns and contribute to changes in rumen VFA. Dietary ingredients can be used to manipulate VFA because changing dietary ingredients alters the type of substrate provided for fermentation (France and Dijkstra, 2005). Although there is considerable information available about how specific starch and fiber sources affect VFA concentrations, Hall et al. (2015) noted that VFA concentrations can be inaccurate representations of treatment effects on fermentation because they do not account corresponding shifts in ruminal liquid volume and passage rate. Liquid passage rate, in particular, has been shown to change with dietary composition, particle size, and other factors (Kuoppala et al., 2009). Although some studies have concurrently evaluated shifts in VFA concentration and fluid passage (e.g., Dijkstra et al., 1993), there has been less focus on how diets with different predicted degradable carbohydrate profiles influence VFA and fluid dynamics.

Rumen liquid volume and passage is quantified using a biological marker (e.g., polyethylene glycol, Cr-EDTA, Co-EDTA) and measuring the exponential decay of the marker over time by taking rumen fluid samples (Krämer et al., 2013). Rumen fluid passage is then used to estimate the amount of VFA exiting the rumen with rumen fluid (fluid-mediated flux) and individual VFA pool size. Fluid dynamics also play a factor in carbohydrate disappearance. Krämer et al. (2013) did not see a difference in ruminal retention time due to fiber type or forage ratio, but did see changes in total mean retention time suggesting rumen liquid outflow could alter nutrient retention times in other portions of the gastrointestinal tract. Increased or decreased nutrient retention times could alter VFA profiles.

Previous modeling work has demonstrated that ruminal starch and fiber disappearance are good predictors of key ruminal factors like microbial N production, efficiency of microbial protein synthesis (Roman Garcia et al. 2016), and ruminal pH (White et al. 2017). Most studies reporting relationships between ruminal VFA and degradable carbohydrate supplies focus exclusively on these aspects of fermentation and do not test the downstream responses of the rumen epithelium or blood metabolites. More holistic analysis of the animal responses to degradable carbohydrate supplies may help more completely characterize how rations differing in nutrient degradability should be formulated to optimize ruminal health and whole-system efficiency.

The objective of this work is to characterize how rumen VFA molar proportions, rumen epithelial gene expression, and blood metabolite concentrations respond to diets containing different starch and fiber sources. We hypothesize that corn grain will have slower starch disappearance than barley grain and will contribute to more balanced fermentation (moderate pH, lower proportion of propionate). We expect timothy hay to have lower fiber disappearance than beet pulp, resulting in lower VFA concentrations and higher proportions of acetate. We expect to detect changes in ruminal VFA transporter gene expression that mirror VFA concentrations. We also expect that shifts in genes associated with epithelial acetate and butyrate metabolism will mirror acetate and butyrate concentrations. Because blood metabolite concentrations are subject to similar fluid kinetic issues as rumen VFA concentrations are (e.g., Hall et al., 2015) blood metabolite concentrations are incomplete representations of metabolite flux (e.g., Bedford et al. In Press). Therefore, we do not expect to detect changes in blood metabolite concentrations that mirror expected shifts in VFA supplies.

MATERIALS AND METHODS

Animals and Treatments

All animal use and procedures were in accordance with Virginia Tech Institutional Animal Care and Use Committee. Six ruminally cannulated yearling Holstein heifers (BW = 330 ± 11.3 kg) were used in a partially replicated 4 x 4 Latin square experiment. Treatment diets were factorially arranged (2 x 2) and were designed to include feedstuffs expected to result in proportionally different supplies of ruminally degradable starch and fiber. Ground barley and ground corn were used as starch sources, with barley expected to generate proportionally faster ruminal starch degradation. Timothy hay and beet pulp were used as fiber sources, with timothy hay expected to have lower ruminal fiber degradability than beet pulp. Corn silage was the diet base, and a mixture of soybean meal, blood meal, and corn gluten feed were added to meet protein requirements and to ensure the diets were isonitrogenous (Table 1). Diets were not designed to be isoenergetic.

Each animal cycled through the four diets over a four-period time frame. Periods consisted of 18 d. The first 3 d were for gradual diet adaptation to prevent rumen upset. Heifers consumed 100% of the treatment diet from d 3 to 18.

Sample Collection

Refusals were collected and heifers were fed via Calan gates (American Calan Inc. Northwood, NH) once per day at approximately 1000 h from d 1 to 13 of each period. From day 14 to 18, heifers were fed every 2 h starting a 1030 h in an attempt to achieve metabolic steady state. Diets were hand-weighed and mixed daily prior to feeding. The amounts of feed offered and refused were weighed daily throughout the experiment and as-fed intake was calculated. Diet and feed refusal samples were collected on d 6, 12, and 18 of each period and were dried in a 55

°C forced-air oven for 48 h to obtain dry matter content of each ration. Daily dry matter intake was calculated by multiplying daily as-fed intake by average dry matter content of each treatment ration per heifer. Body weight was measured at the beginning of the experiment and once between each period (every 18 days). *In situ* degradability of diets was assessed with a 96 h incubation occurring from d 10 to 14 of each period. Rumen fluid samples were collected hourly from 0500 to 2300 h on d 15 through 18 of each period. Blood samples were collected by coccygeal venipuncture at 0900 h on d 18 of each period. Papillae biopsies were collected immediately following blood samples.

Feed Analysis

Diet and refusal samples were dried in a 55 °C forced-air oven (Thermo Scientific Heratherm Advanced Protocol Ovens Model 51028115; Fisher Scientific, Waltham, MA, USA) for 48 h to analyze for DM content. Samples were ground with a Model 4 Wiley mill (A. H. Thomas Scientific, Swedesboro, NJ, USA) to pass through a 1-mm screen. Ash was determined after heating for 8 h in a muffle furnace (500 °C). An Ankom200 fiber analyzer (Ankom Technology, Macedon, NY, USA) with the addition of heat stable α-amylase and sodium sulfite was used to determine NDF. Feed ADF content was assessed using the Ankom200 fiber analyzer (Ankom Technology, Macedon, NY, USA) according to manufacturer specifications. Crude protein was calculated as N x 6.25 after quantification of total N by combustion analysis (Vario El Cube CN analyzer, Elementar Americas Inc., Mount Laurel, NJ, USA). Starch concentrations were determined using the acetate buffer method of Hall (2009) with α-amylase from *Bacillus licheniformis* (FAA, Ankom Technology, Macedon, NY, USA) and amyloglucosidase from *Aspergillus niger* (E-AMGDF, Megazyme International, Wicklow, Ireland).

In Situ Degradability

Diets were prepared according to Table 1, dried for 48 h at 55 ^{o}C, and ground through a 2 mm screen of a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). A total of 40 Dacron bags (Ankom R1020; 10 cm x 20 cm; 50 ± 10 micron porosity; Ankom Technology, Macedon, NY, USA) were filled with 5 g of ground diet samples (DM basis) and heat-sealed. An empty bag was also included for each diet to serve as a blank sample. In situ bags were placed in mesh laundry bags (20 Dacron bags per mesh bag) and incubated in the rumen at: -96 h, -48 h, -24 h, -12 h, -6 h, -3 h, -1.5 h, -1 h, -0.5 h, and 0 h. At time 0 all bags were removed, washed 5 times with a portable washing machine (SKY2767, Best Choice Products, Irvine, CA, USA). Each wash cycle consisted of 1 minute of cool water agitation, drainage, and a 2-minute spin. After washing, bags were dried in a forced-air oven for 48 h at 55 ^{o}C. Bags were immediately weighed post-drying and weighed again after 3 h of air equilibration. Bag contents were then pooled by animal time-point and subjected to the previously described NDF and starch analysis.

Residual amount of each nutrient (NDF and starch) was calculated as the nutrient percentage in each bag multiplied by the DM content in each bag to yield residual grams of nutrient. An exponential decay curve was fitted to the calculated nutrient disappearance (% of total degraded). The slope of this exponential function was multiplied by 100 to yield the fractional nutrient disappearance rate (%/h). A theoretical maximal extent of disappearance was calculated as the nutrient concentration at 96 h minus the nutrient concentration at 0 h divided by the nutrient concentration at time 0, multiplied by 100.

Rumen Fluid Volume, Passage Rate, and VFA Concentrations

A 250 mL polyethylene glycol (PEG; average MW = 8000; Fisher Scientific) intraruminal bolus (38.5 g PEG/250 mL of water) was administered at 0500 h on d 15. The bolus

was administered by funneling solution directly into the rumen through the cannula. Ruminal contents were mixed by hand for 1 minute after the bolus was delivered. Immediately before administration of this bolus, 2 initial (0 h) rumen fluid samples (12 mL per sample) were collected. An additional 0 h sample (12 mL) was collected immediately following the bolus. Rumen fluid samples (12 mL) were then collected hourly until 2300 h. Sample collection on d 16 and 17 also occurred hourly between 0500 and 2300 h, as described above. Rumen fluid samples (12 mL) were collected via polyethylene sampling lines terminating in weighted pot scrubbers, threaded through holes drilled in the rumen cannula cap, and attached to 60 ml syringes. Samples (12 mL) were taken from two locations within the ventral lateral portion of the rumen, pooled in the sampling syringe, and split equally into 3, 5 mL glass vials for storage.

Samples from 0500 to 1700 h of d 15 were analyzed for concentrations of PEG using a protocol modification (M. B. Hall, personal communication) of Smith (1959). Rumen fluid PEG concentrations were fitted to an exponential decay curve over time. The slope of this curve was used to calculate the fractional fluid passage rate (%/h; 100 x slope). The quantity of PEG dosed to each animal was divided by the intercept of the curve to yield an estimate of fluid volume. Fluid volume is better measured other ways (e.g., bailing the rumen) but these approaches were not taken in this study because microbial RNA samples were also collected (data not presented here) and there was concern about whether repeated bailing of the rumen would disrupt microbial populations and functionality.

At h 0700 and 1900, rumen fluid (4 mL) was spot sampled for pH prior to storage. No diurnal patterns were observed in raw rumen pH data obtained from spot sampling and therefore rumen pH data was averaged within day (3 days of measurements per period). All pH

measurements were collected on-farm within 1 minute of sample collection using a calibrated benchtop pH meter (Fisherbrand Accumet AE150; Fisher Scientific).

Rumen fluid samples were also used to measure rumen VFA concentrations by gas chromatography (Firkins et al. 1990). 500 ul from each hourly sample obtained within an animal-period was composited to create 1 sample per animal period that was used for analysis. The rumen VFA pool sizes were estimated as the VFA concentration (mM) multiplied by the rumen fluid pool size (L). The fluid-mediated exit of VFA from the rumen was also estimated as the rumen VFA pool size (mmol) multiplied by the fluid passage rate (%/h).

Blood Metabolite Sample Collection and Analysis

Blood samples were collected from the coccygeal vein at 0900 h on d 18 of each period into 10 ml sodium heparin vacutainer vials (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were kept on ice until plasma isolation. Plasma was isolated by centrifuging blood tubes at 1,500 x g for 10 minutes. Samples were stored at -20°C until VFA derivatization and BHB, glucose, and insulin analysis. Plasma acetate and propionate plasma concentrations were derivatized following (Kristensen, 2000) for gas chromatography mass spectrometry analysis (GCMS). In short, plasma was combined with acetonitrile and 2-chloroethanol before centrifuging at 1,500 x g for 30 min. After centrifuging, the supernatant, NaOH, and heptane were combined allowing phase separation. The bottom phase was removed and transferred to a new vial for the addition of HCl, pyridine, chloroethyl chloroformate, and water. Lastly, chloroform was added to complete derivatization. 0.80 ul samples were analyzed on a Thermo Electron Focus Gas Chromatograph (Thermo Scientific, Waltham, MA, USA) working with a Thermo Electron Polaris Q mass spectrometer (MS; Thermo Scientific, Waltham, MA, USA) and XCalibur software (version 1.4; Thermo Scientific). Plasma BHB concentrations were

measured using B-hydroxybutyrate (Ketone Body) Colorimetric Assay Kit (Cayman Chemical Item No. 700190, Cayman Chemical, Ann Arbor, MI). Plasma glucose concentrations were measured using StanbioTM Glucose Liquid Reagent for Diagnostic Set (Fisher Scientific SB-1070-125). Plasma insulin concentrations were measured using a chemiluminescence assay (Siemens Immulite 2000 XPi Immunoassay System, Siemens Healthcare, CA, USA).

Papillae Biopsy Sample Collection

Rumen papillae samples were collected on d 18 immediately following blood collection. During papillae biopsies, heifers were restrained in a head gate and working chute. Biopsies were collected by cutting 60 to 80 papillae from the ventral surface of the epithelium using cuticle scissors. To access the epithelium, an assistant firmly grasped the ventral sac of the rumen and inverted the epithelium through the cannula, exposing it for sampling. Prior to sampling, papillae were cleaned with 7.0 (pH) phosphate buffered saline (Potassium Phosphate monobasic 210 mg/L; Sodium Chloride 9000mg/L; Sodium Phosphate dibasic, 726 mg/L; Gibco Life Technologies, Grand Island, NY, USA). Upon severing the papillae from the epithelium, papillae were immediately immersed in an RNA preservative solution (RNALater, QIAGEN; Valencia, CA, USA). After all papillae were collected, papillae and solution were transferred to 2 cryovials and stored at -80°C until RNA was isolated using an RNA Plus Mini Kit (QIAGEN; Valencia, CA, USA). cDNA was synthesized using the Applied Biosystem High-Capacity cDNA Reverse Transcription Kit (ThermoFischer Scientific; Waltham, MA, USA) and a mastercycler. Real-Time reverse transcription quantitative PCR (Real-Time qRTPCR; method described by Ealy et al., 2017) was conducted to test several candidate housekeeper genes: ribosomal protein S9, S15 and S26. This method used ABI MicroAmp Fast Optical 96-well reaction plates (0.1 ml; Fisher 43-469-07, Fisher Scientific) to hold 1 ul of sample (or housekeeper) cDNA in combination with a

primer “Master Mix” for analysis by the Real-Time Fast machine (Applied Biosystems™ 7500 Real-Time PCR; ThermoFischer Scientific, Waltham, MA, USA). The “Master Mix” included 5 ul of SYBR Fast Green Master Mix (Fisher 43-856-12, Fisher Scientific), 0.5 ul reverse and 0.5 ul of forward primers of the target gene, and 3 ul molecular grade water. The calculated 2(-Delta Delta cycle threshold) (C(T)) values for these potential housekeeper genes were evaluated. Ribosomal protein S15 (RPS15) had the highest average 2(-Delta Delta C(T)) value making it the best option as a housekeeper. As such, it was used for all subsequent analyses. The housekeeper was plated with each triplicate of genes to serve as a standard. The same Real-Time qRTPCR protocol (Ealy et al., 2017) was used for individual gene analysis. 14 genes were chosen for analysis because of their roles in rumen epithelial transport, epithelial integrity, or VFA metabolism. Genes included: monocarboxylate transporters 1, 2, and 4 (MCT1, MCT2, MCT4), sodium-hydrogen exchanger isoforms 1, 2, and 3 (NHE1, NHE2, NHE3), Claudin-1 (CLDN1), gap-junction protein alpha 1 (GJA1), acetoacetyl-CoA synthetase (AACS), 3-hydroxybutyrate dehydrogenase type 1 (BDH1), 3-hydroxymethyl-3-methylglutaryl-CoA lyase (HMGCL), 3-hydroxy-3-methylglutaryl (HMGCS2), heat shock protein 70 (HSP70), and serine-threonine protein kinase (AKT1).

Calculations and Statistical Analysis

All statistical analyses were conducted using the nlme package of R version 3.1.0. (R Core Team, 2014). Dry matter intake (kg/d), average daily gain (kg/d), VFA concentrations (mM), VFA molar proportions (% of total VFA), fluid volume (L), fluid passage rate (%/h), calculated VFA pool sizes (mmol), calculated VFA fluid exit rates (mmol), epithelial gene expression (2(-Delta Delta C(T))), and blood metabolite concentrations (mM, mg/dL, or uIU/mL) were considered as

response variables. The following linear mixed effect model was used for analysis of all response variables:

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

where μ is the overall mean, α_i is the effect of the i^{th} starch source, β_j is the effect of the j^{th} fiber source, $\alpha\beta_{ij}$ is the interaction of starch 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 starch source *i*, fiber source *j*, animal *k*, and period *l*.

For each response variable, different residual error variance structures (Unstructured, Compound Symmetry, and 1st Order Autoregressive) were compared and the model with the lowest Akaike information criterion (AIC; 1st Order Autoregressive in all cases) was used to test significance. Analysis of variance and least square means were computed for each model. Despite a prevailing lack of significant interactions, least square means for each treatment combination are presented to enable future model-based work, which may benefit from these treatment-specific values. Treatment effects were considered significant at $P \leq 0.05$ and tendencies at $0.05 \leq P \geq 0.15$.

RESULTS AND DISCUSSION

Dry Matter Intake

Average DMI for all treatments throughout all periods is shown in Figure 2. Dry matter intake was affected by fiber source ($P = 0.009$) but was unaffected by starch source ($P = 0.29$) or the interaction between fiber and starch sources ($P = 0.46$). Heifers fed beet pulp diets consumed less than heifers on timothy hay diets (Figure 2). There are several reasons why DMI may have differed among treatments. First, it is possible that heifers found beet pulp to be less palatable than timothy hay. We believe palatability may have been an issue because the beet pulp had to be soaked

in water prior to ration mixing to entice some animals to consume it. Secondly, it is also possible that the higher expected energy concentration of beet pulp diets (Estimated ME, Mcal/kg 2.74 to 2.75; Table 1) caused lower DMI. The more degradable fiber in the beet pulp diets may have contributed to enhanced hepatic oxidation of VFA and subsequent activation of satiety feedback mechanisms in the brain (Allen et al., 2009). Allen (2000) demonstrated highly fermentable starch diets decreased DMI, which suggests propionate metabolism provides satiety feedback to the brain (Allen et al., 2009). Although this dietary regulation of feed intake is most commonly associated with starch degradation, we did not expect starch degradability to influence feed intake in this study because the extent of starch degradation did not differ among diets, only the rate of starch disappearance.

Average Daily Gain

Heifer ADG is shown in Figure 2. Treatments did not affect ADG (Starch $P = 0.49$; Fiber $P = 0.50$; Starch x Fiber $P = 0.63$). The lack of ADG response is interesting because diets differ in predicted ME concentration but not in nutrient composition (Table 1). The estimated ME values for each treatment diet ranged from 2.62 to 2.75 Mcal/kg and are greater than the NRC recommended ME value of 2.28 Mcal/kg for 350 kg Holstein heifers at 12 months of age (Table 14-16; NRC, 2001). The greater estimated ME values for the treatment diets suggests energy should not have limited growth. This lack of energy limitation on growth is perhaps why the diets differing in ME content did not result in differences in ADG throughout the experimental period. Additionally, we would not expect the number of animals reported here would be sufficient to detect changes in growth parameters.

Nutrient Degradability

Starch and fiber rate and extent of disappearance results for each treatment diet are shown in Figure 3. Starch source affected the rate of starch disappearance ($P = 0.05$) but not the extent of disappearance ($P = 0.25$). Fiber source did not affect starch disappearance rate ($P = 0.58$) or extent of disappearance ($P = 0.42$) These results were expected because the rumen is the primary location for starch degradation regardless of the ingredient being described as highly or lowly degradable in the rumen. Although starch from barley and corn has been thought to have different ruminal extent of disappearance (Ferreira et al., 2018), the lack of statistical difference in extent of disappearance could be explained by similar proline content of the endosperm (Kung et al., 2014) or lack of variation in endosperm vitreousness between these sources of cereal grain (Ferreira et al., 2018).

Corn diets degraded faster (7.61 to 10.5 %/h) than barley diets (7.30 to 8.52 %/h; Figure 3) which was not expected. The difference in disappearance rate contradicts Ferreira et al. (2018) which showed corn grain to have a slower disappearance rate than hulled barley. The corn fed to heifers in the present study was not pelleted and did not go through any high-heat processing. It is possible that this lack of processing contributed to the faster degradation. The differences between these studies may be due to testing dietary starch disappearance versus individual ingredient starch disappearance. Additional work is needed to explain why corn-based diets in the present study had faster starch disappearance rates than corresponding barley diets.

Calculated fiber disappearance rate was not influenced by starch source ($P = 0.98$), fiber source ($P = 0.21$), or the interaction of starch and fiber sources ($P = 0.47$). The corn and beet pulp diet resulted in the numerically fastest fiber disappearance rate, perhaps reflecting an optimized pairing of starch and fiber sources. The corn and timothy hay diets resulted in the lowest

disappearance rate. Varga and Hoover (1983) did not statistically compare timothy hay and beet pulp degradation rates but reported close values of 6.2 %/h and 5.5 %/h, respectively, which agreed with the lack of difference in disappearance rate observed in the present work.

Extent of fiber disappearance was impacted by fiber source ($P = 0.0007$) and starch source ($P = 0.0009$). The interaction of starch and fiber source tended to ($P = 0.11$) influence fiber extent of disappearance. Beet pulp had higher extent of fiber disappearance than timothy hay diets. This was expected because beet pulp is a more rapidly degradable fiber source compared to other by-product feedstuffs (DePeters et al., 1997; Fadel et al., 2000) and timothy hay is known to have a thicker, denser cell wall than beet pulp (Van Soest et al., 1991). Varga and Hoover (1983) reported different extents of degradation for beet pulp (68.9 % total NDF) and timothy (47.1 % total NDF), which are similar to the values calculated in the present study.

Rumen pH

Rumen pH responses are presented in Figure 4. Rumen pH was affected by the fiber source used in treatment diets (Starch $P = 0.13$; Fiber $P = 0.02$; Starch x Fiber $P = 0.18$). Heifers consuming timothy hay diets had higher ruminal pH in comparison to beet pulp diets (Figure 4). This result was expected because the role of forage NDF in maintaining rumen health has been well-characterized (White et al., 2017a; White et al., 2017b). The more soluble fiber present in beet pulp is not expected to have the same buffering characteristics as forage NDF. The lack of a starch source effect is understandable because the extent of starch disappearance was not different among starch sources. Additionally, by feeding every 2 h diurnal pH fluctuations associated with different rates of starch disappearance could have been minimized.

Volatile Fatty Acid Molar Proportions and Concentrations

Treatment means and *P*-values for rumen VFA molar proportions and concentrations are shown in Tables 2 and 3, respectively. The VFA concentrations are reported for coherence with the previous literature only. Hall et al. (2015) demonstrated that concentrations are unreliable representations of treatment effects because they fail to account for fluid pool size, absorption, or passage. As such, only molar proportions, and calculated total VFA pool size and fluid mediated fluxes are discussed. Isovalerate (% mol) was the only rumen VFA to be influenced by a starch by fiber interaction ($P = 0.05$). Heifers consuming the C-TH diet had the highest molar proportion of isovalerate, contrastingly, heifers consuming the C-BP diet had the lowest molar proportion of isovalerate. The review by Andries et al. (1987) suggests the catabolism of amino acids is a primary cause of branched chain volatile fatty acid (**BCVFA**) production. The inconsistent fluctuations of isovalerate molar percentage could be explained by varying ruminal protein degradability within the individual feedstuffs (corn versus barley; TH versus BP) even though diets were formulated to be isonitrogenous. Molar proportions of ruminal acetate ($P = 0.02$) and isovalerate ($P = 0.008$) were greater when animals were fed timothy hay diets in comparison to BP diets. Ruminal propionate ($P = 0.06$) and valerate ($P = 0.10$) molar proportions also tended to increase when animals were fed BP diets in comparison to TH diets. This shift in VFA molar proportions from an acetate-based fermentation towards increased proportions of propionate and valerate is likely due to the increased ruminal degradability of NDF on the BP diets. Ruminal propionate ($P = 0.02$) and valerate ($P = 0.049$) molar percentages were decreased on barley diets, but butyrate molar percentage increased ($P = 0.04$) in comparison to corn diet counterparts. The shift from propionate and valerate to increased

butyrate may have been due to the slower apparent starch degradation rate of barley- versus corn-based diets.

Rumen Fluid Volume

Estimated rumen fluid pool size (rumen fluid volume) and rumen fluid passage rate are shown in Figure 5. Both estimated rumen fluid volume (L) (Starch $P = 0.31$; Fiber $P = 0.75$; Starch x Fiber $P = 0.55$) and estimated rate of passage (%/h) (Starch $P = 0.68$; Fiber $P = 0.80$; Starch x Fiber $P = 0.30$) were unaffected by treatment (Figure 5). Fluid volume and passage rate are dependent upon water intake and osmolality. Water intake can be influenced by dry matter intake (Little and Shaw, 1978); however, the significant fiber effect on DMI observed in this study did not translate to significant differences in fluid volume or passage rate. Osmolality of rumen fluid was not measured in this study but it is possible that shifts in osmolality offset any potential shifts in water intake associated with different DMI among diets. Conversely, it is possible that the differences in water intake and osmolality among diets were insufficient to drive changes in fluid volume and passage rate. Due to the similarity among diets, we believe the latter is more likely.

Estimated rumen fluid passage (L/h) was also calculated by multiplying estimated rumen fluid volume by estimated rate of passage. Estimated rate of passage (L/h) was also unaffected by treatment (Starch $P = 0.31$; Fiber $P = 0.52$; Starch x Fiber $P = 0.23$). Although not statistically significant, the fluid passage (L/h) data followed a similar pattern to the extent of fiber degradation data, where the BP-T diet was different from the other diets, suggesting a possible relationship between the extent of fiber degradation and the fluid movement from the rumen.

Individual VFA Pool Size and Calculated Fluid-Mediated Flux

Individual VFA pool size and calculated fluid-mediated flux (from the rumen through liquid passage) are shown in Table 4. None of the estimated VFA pool sizes were affected by diet ($P > 0.15$). This lack of significance further highlights the need to evaluate fermentations in the context of fluid dynamics. If we had evaluated differences based on concentration or molar proportion alone, the results may have suggested greater differences among diets than are implied based on this pool-size analysis.

In contrast to the lack of change in fluid passage rate, TH diets significantly increased isovalerate passage from the rumen with the fluid fraction ($P = 0.05$). Barley also tended to result in greater fluid-mediated exit of isovalerate ($P = 0.13$) and butyrate ($P = 0.14$) from the rumen. These results are a direct product of numerical differences in isovalerate and butyrate concentrations among diets and fluid passage rates. Interpreting the biological significance of fluid-mediated VFA exit from the rumen is difficult because most of this VFA is likely absorbed in downstream portions of the gastrointestinal tract. Thus, its difference from VFA absorbed directly from the rumen is likely minimal. Future work quantifying post-ruminal VFA absorption may help clarify the biological importance of this flux.

Gene Expression

Gene name, abbreviation, function, starch source, fiber source, and starch by fiber interaction *P*-values are presented in Table 5. Beet pulp tended to promote expression of the BDH1 gene ($P = 0.08$). This shift in BDH1 expression is likely due to the tendency for higher butyrate concentrations and molar proportions on BP diets. Increased butyrate availability will likely drive metabolism of butyrate in the rumen epithelium, resulting in increased expression of genes involved in that metabolic pathway. Not all genes involved in butyrate metabolism were differentially expressed on BP diets. This inconsistency may reflect insufficient variation among

responses to reliably detect differences between treatments or could indicate that BDH1 is more rate-limiting that other steps in this pathway.

Sodium hydrogen exchanger isoform 3 expression was higher on TH diets than on BP diets ($P = 0.05$) (Table 5). This result is consistent with the rumen pH data, which showed higher pH on TH diets than on BP diets (Figure 4). Sodium hydrogen exchanger isoform 3 (Figure 1) helps maintain epithelium homeostasis by transporting sodium into the cell, and transporting protons into the rumen. The activation of this gene may reflect increased pressure to maintain both ruminal and epithelial homeostasis on diets with differing pH, as has been suggested by Steele et al. (2011).

Monocarboxylate transporter isoform 4 tended to be affected by fiber source, with increased relative expression on TH diets ($P = 0.06$) (Table 5). Monocarboxylate transporter isoform 4 is responsible for short chain fatty acid (SCFA) transport across the rumen epithelium and maintenance of epithelium ion homeostasis (Figure 1). It was expected that the apical-most transporters (MCT4 and NHE3) would be affected by diet because they would have the most prolonged and abundant contact with feed. However, it was also expected that NHE1 and MCT1 would be affected by treatment because the two types of transporters tend to work in tandem with different isoforms. The lack of change in NHE1 and MCT1 does support the theory that strata position of the transporter may be a driving force in regulation of expression.

Timothy hay diets increased expression of HSP70 in comparison to BP counterparts ($P = 0.02$). Heat shock protein-70 connections to heat stress have been investigated in ruminants and HSP70 is responsible for the folding and unfolding of other proteins particularly under thermal stress conditions to prevent protein aggregation (Mishra and Palai, 2014). Heat shock protein-70 also has shown to be maximally expressed at a rumen pH of 6.0 to 6.4 in goats (Hollman et al., 2013). Timothy hay diets had greater average rumen pH in comparison to their BP counterparts

(Figure 4). It is possible TH diets fostered an environment for HSP70 to be upregulated. Hollman et al. (2013) also investigated the impacts of high energy diets (i.e. rapidly fermentable diets) on HSP70 expression, which was lowly expressed at the protein level used in their treatments. In the present study, there was no starch source effect on HSP70 suggesting high energy feedstuffs did not drive HSP70 expression at the gene level. Additional research is needed to understand why these diets caused shifts in HSP70.

Blood Metabolites

Table 6 includes plasma concentrations of acetate, propionate, BHB, glucose, and insulin of the different treatments and the treatment *P*-values. Acetate was the only metabolite that tended to be affected by treatment (Starch x Fiber $P = 0.067$). It was unexpected that acetate concentrations would be changed due to treatment because we expected blood acetate to be utilized by peripheral tissues and regulated to some homeostasis before samples were taken at the tail vein. The B-BP diet had the greatest blood acetate concentration and the C-BP diet had the lowest blood acetate concentration. The direction and magnitude of blood acetate differences does not map to VFA pool size data, VFA concentration data, or VFA molar proportions. This discrepancy highlights challenges with using blood VFA concentrations as a means of representing changes in metabolite availability associated with treatment. Future work is needed to more thoroughly understand the relationships between post-absorptive VFA supply and blood VFA concentrations.

Plasma propionate, BHB, glucose, and insulin concentrations were unaffected by starch or fiber source (Table 6). It was expected that blood propionate concentrations would not change because propionate is most likely metabolized by the liver prior to sample collection. Previous work measuring blood propionate concentrations during a jugular propionate infusion also found that shifts in propionate concentration are not sustained, indicating homeostatic mechanisms to

maintain blood propionate concentration (Bedford et al., In Press). Like ruminal VFA concentrations, blood VFA concentrations only provide a snapshot of biological responses (Hall et al., 2015). Therefore, additional flux-based work is needed to more completely understand blood VFA metabolism.

Conclusions

This study demonstrated that nutrient degradability is a factor driving VFA dynamics and subsequent epithelial responses. The fiber sources used in this study generated more measurable shifts in fermentation than the starch sources. Additional work on starch sources differing in extent of degradation is needed to understand whether this relative importance is simply reflecting the relative importance of extent of ruminal degradation versus rate of degradation. The work also provides further evidence for the need for more holistic evaluation of how diets influence the fermentation environment of the rumen, including the epithelium.

Acknowledgements

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Figures

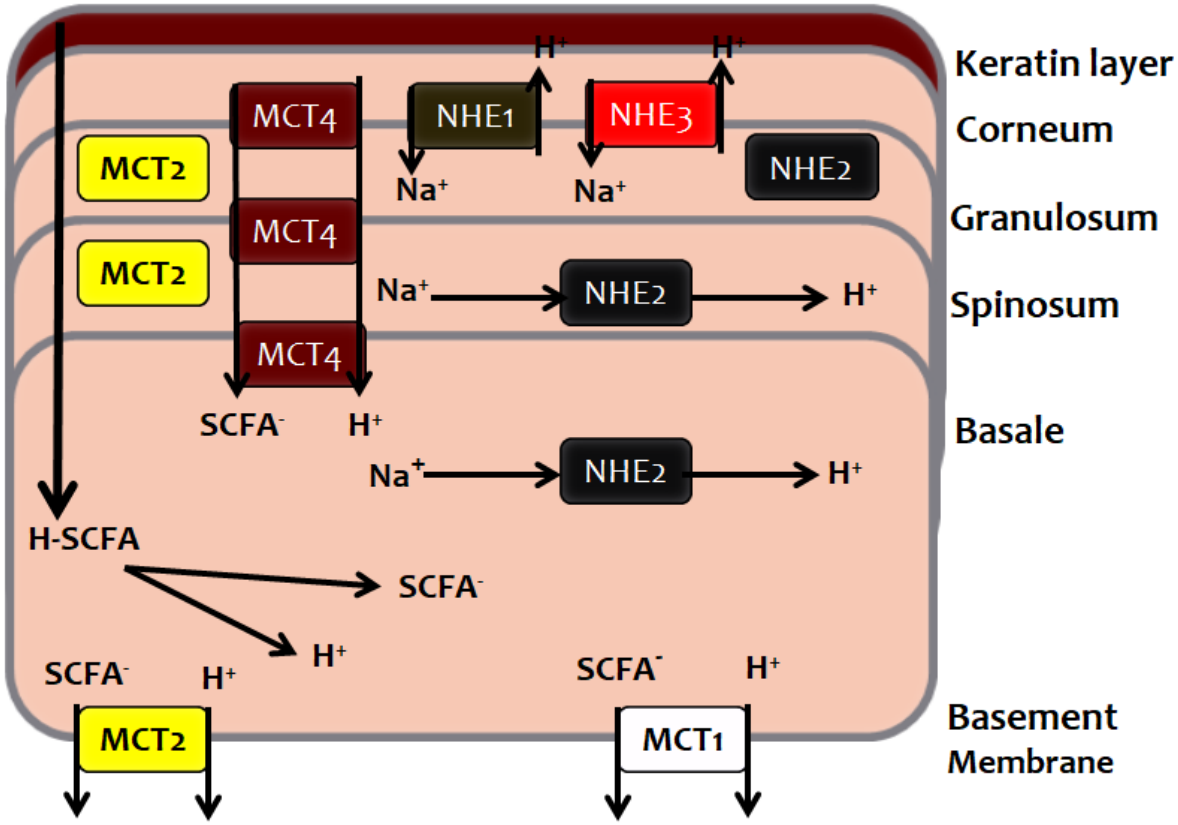

Figure 3.1: This figure was adapted from Laarman et al. (2016) and Yohe et al. (unpublished data). This figure describes the function, placement, and location of the rumen epithelium short chain fatty acid transporters and sodium hydrogen exchangers.

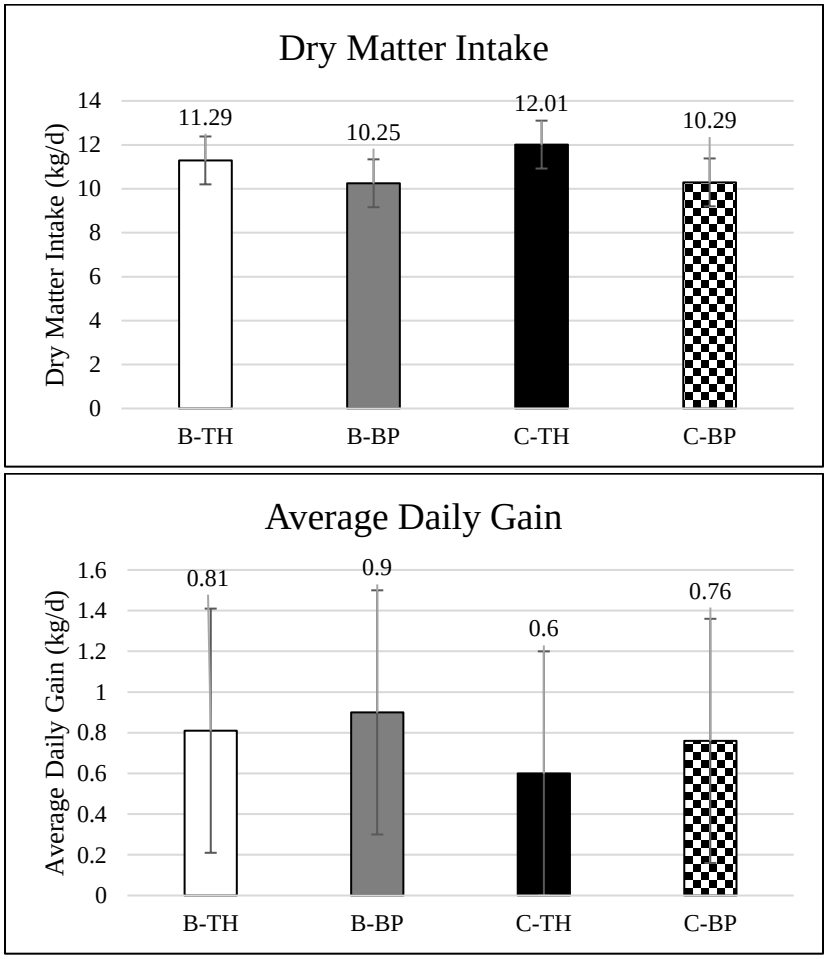

Figure 3.2: Dry matter intake in kg/d for each treatment diet is shown in the first chart (Starch *P* = 0.29; Fiber *P* = 0.009; Starch x Fiber *P* = 0.46). Average daily gain in kg/d for each treatment diet is shown in the second chart (Starch *P* = 0.74; Fiber *P* = 0.41; Starch x Fiber *P* = 0.88). The minimum and maximum average daily gain ranged from 0.60 kg/d to 0.90 kg/day for all the treatments, and some animals were either significantly below or above these averages causing a large SE. Significance is determined by $P < 0.05$ and tendency by $P < 0.15$.
B-TH: Ground Barley and Timothy Hay Pellets
B-BP: Ground Barley and Beet Pulp Pellets
C-TH: Ground Corn and Timothy Hay Pellets
C-BP: Ground Corn and Beet Pulp Pellets

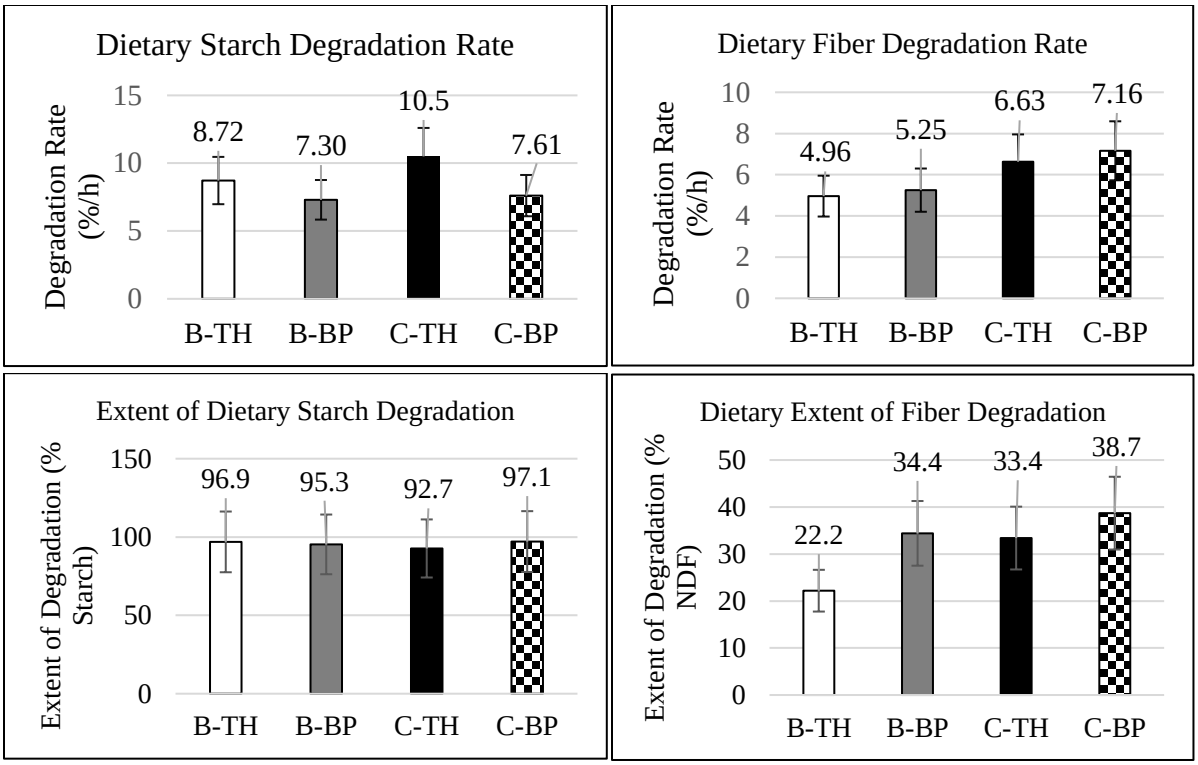

Figure 3.3: Starch and fiber ruminal degradation rate and extent of degradation. Degradation rate is represented in %/h and extent of degradation is represented in % nutrient (starch or NDF). Corn diets had faster starch degradation rates than barley diets (Starch $P = 0.05$). Timothy hay diets did not differ from beet pulp diets for starch degradation rate (Fiber $P = 0.58$; Starch x Fiber $P = 0.34$) and extent of starch degradation (Starch $P = 0.25$; Fiber $P = 0.42$; Starch x Fiber $P = 0.59$). Beet pulp diets had greater extent of fiber degradation (Fiber $P = 0.0007$) and corn diets had greater extent of fiber degradation (Starch $P = 0.0009$; Starch x Fiber $P = 0.11$). Diets did not differ by fiber degradation rate (Starch $P = 0.98$; Fiber $P = 0.21$; Starch x Fiber $P = 0.47$). Significance is determined by $P < 0.05$ and tendency by $P < 0.15$.
B-TH: Ground Barley and Timothy Hay Pellets
B-BP: Ground Barley and Beet Pulp Pellets
C-TH: Ground Corn and Timothy Hay Pellets
C-BP: Ground Corn and Beet Pulp Pellets

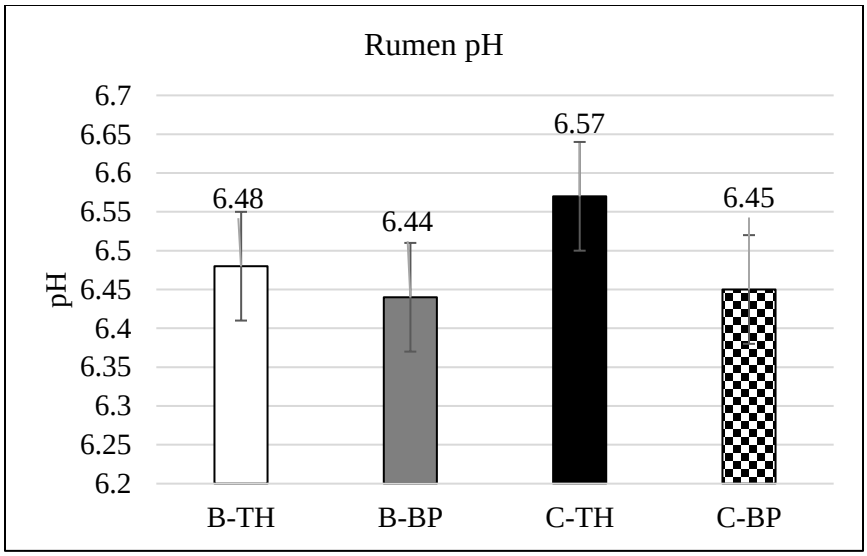

Figure 3.4: Average rumen pH for each treatment diet. Ruminal pH was spot sampled every six hours during d 15 to d 17 of each period. Timothy hay diets had greater rumen pH than beet pulp diets ($P = 0.02$). Corn diets tended to have greater rumen pH ($P = 0.13$) than barley diets. There was no interaction effect (Starch x Fiber $P = 0.18$). Significance is determined by $P < 0.05$ and tendency by $P < 0.15$.
B-TH: Ground Barley and Timothy Hay Pellets
B-BP: Ground Barley and Beet Pulp Pellets
C-TH: Ground Corn and Timothy Hay Pellets
C-BP: Ground Corn and Beet Pulp Pellets

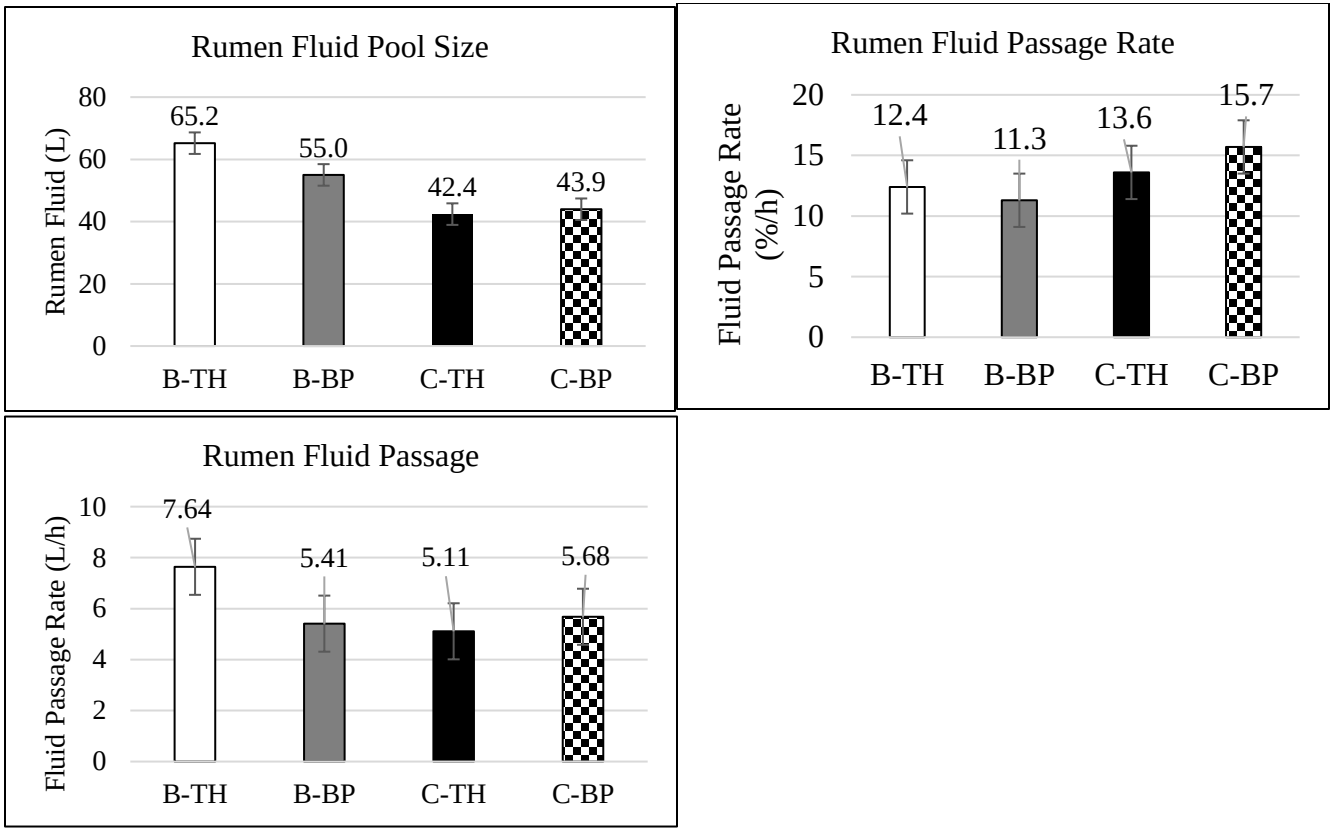

Figure 3.5: Rumen fluid pool size (first chart) describes the amount of fluid volume in the rumen in liters. Treatment diets did not differ in rumen fluid pool size (Starch $P = 0.31$; Fiber $P = 0.75$; Starch x Fiber $P = 0.55$). Rumen fluid passage rate (second chart) describes the rate of fluid exit out of the rumen in percent per hour (Starch $P = 0.68$; Fiber $P = 0.80$; Starch x Fiber $P = 0.30$). Rumen fluid passage (third chart) describes the rate of fluid exit out of the rumen in liters per hour (Starch $P = 0.31$; Fiber $P = 0.52$; Starch x Fiber $P = 0.23$). Significance is determined by $P < 0.05$ and tendency by $P < 0.15$.
B-TH: Ground Barley and Timothy Hay Pellets
B-BP: Ground Barley and Beet Pulp Pellets
C-TH: Ground Corn and Timothy Hay Pellets
C-BP: Ground Corn and Beet Pulp Pellets

Tables

Table 3.1: Diet ingredient and nutrient inclusion for each treatment diet.

Ingredients, % of DM	**B-TH[1]**	**B-BP[2]**	**C-TH[3]**	**C-BP[4]**
Timothy Hay	19.9	0.02	25.0	1.43
Beet Pulp	6.05	30.2	3.55	32.9
Ground Corn	0.38	0.00	10.5	12.3
Ground Barley	14.1	14.8	0.67	0.31
Corn Silage	34.4	35.6	35.2	29.4
Corn Gluten Feed	3.26	0.00	1.65	7.22
Soybean meal	9.30	17.1	9.95	15.3
Blood Meal	3.78	0.00	4.37	0.04
Nutrient, % of DM[5]				
DM	64.8	73.4	63.9	74.7
NDF	35.0	35.0	35.0	35.0
Starch	20.0	20.0	20.0	20.0
CP	15.9	16.0	15.9	16.0
ME, Mcal/kg	2.63	2.74	2.62	2.75

[1]B-TH: Ground Barley and Timothy Hay Pellets

[2]B-BP: Ground Barley and Beet Pulp Pellets

[3]C-TH: Ground Corn and Timothy Hay Pellets

[4]C-BP: Ground Corn and Beet Pulp Pellets

[5]Diets were formulated to have similar nutrient inclusion (% of DM).

Table 3.2: Calculated VFA molar proportions for animals receiving diets differing in expected ruminal starch and fiber degradation rate and extent of degradation. Starch ingredients included barley and corn and fiber ingredients included timothy hay and beet pulp.

	Treatment					*P*-values[5]		
Molar Percentage, % mol	**B-TH[1]**	**B-BP[2]**	**C-TH[3]**	**C-BP[4]**	**SEM**	**Fiber**	**Starch**	**Starch x Fiber**
Acetate	64.1	63.4	63.8	62.6	0.77	0.02	0.12	0.50
Propionate	17.3	17.5	17.6	18.9	0.77	0.06	0.02	0.17
Butyrate	15.0	15.4	14.7	15.0	0.85	0.15	0.04	0.85
Valerate	1.30	1.39	1.38	1.44	0.11	0.10	0.049	0.77
Isobutyrate	0.86	0.85	0.87	0.77	0.08	0.13	0.22	0.25
Isovalerate	0.55	0.53	0.58	0.5	0.0	0.008	0.27	0.05
2-MethylButyrate	0.98	0.96	0.93	0.91	0.29	0.89	0.69	0.99

[1]B-TH: Ground Barley and Timothy Hay Pellets

[2]B-BP: Ground Barley and Beet Pulp Pellets

[3]C-TH: Ground Corn and Timothy Hay Pellets

[4]C-BP: Ground Corn and Beet Pulp Pellets

[5]Significance is determined by $P < 0.05$ and tendency by $P < 0.15$.

Table 3.3: VFA concentrations for animals receiving diets differing in expected ruminal starch and fiber degradation rate and extent of degradation. Starch ingredients included barley and corn and fiber ingredients included timothy hay and beet pulp.

	Treatment					*P*-values[5]		
VFA Concentration, mM	**B-TH[1]**	**B-BP[2]**	**C-TH[3]**	**C-BP[4]**	**SEM**	**Fiber**	**Starch**	**Starch x Fiber**
Acetate	82.5	79.8	87.0	71.2	8.24	0.009	0.37	0.05
Propionate	22.2	21.8	24.2	21.8	1.87	0.16	0.26	0.28
Butyrate	19.4	19.5	19.8	17.2	2.43	0.14	0.12	0.12
Valerate	1.67	1.76	1.88	1.67	0.28	0.014	0.25	0.05
Isobutyrate	1.10	1.06	1.19	0.85	0.18	0.0031	0.36	0.02
Isovalerate	0.70	0.66	0.80	0.50	0.16	0.47	0.28	0.58
2-MethylButyrate	1.29	1.19	1.29	1.01	0.46	0.28	0.47	0.58
Total VFA	129	126	136	114	14.8	0.02	0.49	0.06
Total BCVFA	4.80	4.70	5.09	4.03	0.79	0.04	0.32	0.09

[1]B-TH: Ground Barley and Timothy Hay Pellets
[2]B-BP: Ground Barley and Beet Pulp Pellets
[3]C-TH: Ground Corn and Timothy Hay Pellets
[4]C-BP: Ground Corn and Beet Pulp Pellets
[5]Significance was determined for a $P < 0.05$ and a tendency $P < 0.15$.

Table 3.4: Calculated fluid-mediated VFA flux and ruminal VFA pool size for animals receiving diets differing in expected ruminal starch and fiber degradation rate and extent of degradation. Starch ingredients included barley and corn and fiber ingredients included timothy hay and beet pulp.

Fluid-Mediated VFA Flux (mmol/h)[5]	**Treatments**					***P*-values[7]**		
VFA	**B-TH[1]**	**B-BP[2]**	**C-TH[3]**	**C-BP[4]**	**SEM**	**Fiber**	**Starch**	**Starch x Fiber**
Acetate	647	440	425	418	90.5	0.27	0.18	0.29
Propionate	171	123	117	126	25.0	0.47	0.33	0.27
Butyrate	156	111	95.9	102	22.0	0.46	0.14	0.28
Valerate	12.8	9.56	8.81	9.58	1.80	0.52	0.31	0.28
Isobutyrate	8.41	5.61	5.76	4.89	1.20	0.16	0.18	0.45
Isovalerate	5.29	3.47	3.81	2.74	0.71	0.05	0.13	0.61
2-Methylbutyrate	9.67	6.62	5.91	6.22	1.58	0.45	0.27	0.31
VFA Pool Size (mmol)[6]								
VFA	**B-TH[1]**	**B-BP[2]**	**C-TH[3]**	**C-BP[4]**	**SEM**	**Fiber**	**Starch**	**Starch x Fiber**
Acetate	5640	4450	3410	3170	905	0.48	0.17	0.60
Propionate	1490	1220	924	950	236	0.67	0.19	0.54
Butyrate	1330	1130	791	782	235	0.68	0.22	0.65
Valerate	108	96.0	71.7	71.4	19.8	0.74	0.26	0.72
Isobutyrate	67.7	58.7	48.5	37.1	12.5	0.39	0.26	0.92
Isovalerate	42.0	36.0	32.3	21.4	7.50	0.22	0.30	0.70
2-Methylbutyrate	79.6	60.4	47.8	49.5	12.7	0.55	0.41	0.40

[1]B-TH: Ground Barley and Timothy Hay Pellets
[2]B-BP: Ground Barley and Beet Pulp Pellets
[3]C-TH: Ground Corn and Timothy Hay Pellets
[4]C-BP: Ground Corn and Beet Pulp Pellets
[5]Volatile fatty acid flux is equivalent to VFA escaping the rumen with fluid passage.

[6]Volatile fatty acid pool size describes the amount present as a function of rumen liquid volume.
[7]Significance was determined with a $P < 0.05$ and a tendency $P < 0.15$.

Table 3.5: Gene name, abbreviation, function, and starch, fiber, and starch by fiber *P*-values for each gene investigated when animals were fed diet differing in expected fiber and starch degradation rate and extent of degradation.

			***P*-values[1]**		
Gene Name	**Abbreviation**	**Function**	**Fiber**	**Starch**	**Starch x Fiber**
Acetoacetyl-CoA Synthetase	AACS	Converts acetoacetate to acetoacetyl-CoA	0.17	0.35	0.39
Beta-Hydroxybutyrate Dehydrogenase	BDH1	Converts acetoacetate to beta-hydroxybutyrate	0.08	0.62	0.14
3-Hydroxymethyl-3-Methylglutaryl-CoA Lyase	HMGCL	Converts 3-hydroxy-3-methylglutaryl-CoA to acetyl-CoA and acetoacetate.	0.44	0.26	0.16
3-Hydroxy-3-Methylglutaryl-CoA Synthase 2	HMGCS2	Converts acetoacetyl-CoA to HMG-CoA	0.79	0.37	0.67
Serine-Threonine Protein Kinase B	AKT1	mTOR signaling; cell growth and proliferation	0.16	0.16	0.17
Heat Shock Protein 70	HSP70	protein folding and unfolding	0.05	0.44	0.45
Claudin-1	CLDN1	Properties of epithelial tight junctions	0.21	0.77	0.51
Gap Junction Protein Alpha 1	GJA1	Encodes Connexin proteins	0.49	0.38	0.41
Monocarboxylate Transporter Isoform 1	MCT1	Short chain fatty acid and proton symporter	0.09	0.95	0.69
Monocarboxylate Transporter Isoform 2	MCT2	Short chain fatty acid symporter	0.18	0.20	0.58
Monocarboxylate Transporter Isoform 4	MCT4	Short chain fatty acid and proton symporter	0.06	0.15	0.19
Sodium Hydrogen Exchanger Isoform 1	NHE1	Sodium hydrogen antiporter	0.77	0.97	0.69
Sodium Hydrogen Exchanger Isoform 2	NHE2	Intracellular sodium hydrogen exchanger	0.27	0.71	0.52
Sodium Hydrogen Exchanger Isoform 3	NHE3	Sodium hydrogen antiporter	0.05	0.32	0.12

[1]Significance was determined with $P < 0.05$ and a tendency $P < 0.15$.

Table 3.6: Blood plasma metabolite concentrations for each treatment diet, which were formulated based on expected ruminal starch and fiber degradation rate and extent of degradation. Acetate, propionate, and beta-hydroxybutyrate are presented in mM, glucose in mg/dL, and insulin uIU/mL.

	Treatments					***P*-value[5]**		
Metabolite	**B-TH[1]**	**B-BP[2]**	**C-TH[3]**	**C-BP[4]**	**SEM**	**Fiber**	**Starch**	**Starch x Fiber**
Acetate, mM	0.73	1.04	0.87	0.49	0.21	0.71	0.16	0.067
Propionate, mM	0.076	0.079	0.078	0.074	0.005	0.99	0.77	0.46
Beta-hydroxybutyrate, mM	0.25	0.33	0.35	0.34	0.10	0.33	0.24	0.31
Glucose, mg/dL	86.0	85.9	79.4	62.7	13.5	0.22	0.18	0.47
Insulin, uIU/mL	15.7	14.7	12.2	16.5	2.91	0.47	0.80	0.28

[1]B-TH: Ground Barley and Timothy Hay Pellets
[2]B-BP: Ground Barley and Beet Pulp Pellets
[3]C-TH: Ground Corn and Timothy Hay Pellets
[4]C-BP: Ground Corn and Beet Pulp Pellets
[5]Significance was determined with $P < 0.05$ and a tendency $P < 0.15$.

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GENERAL CONCLUSION

The overarching goal to this work was to investigate different ways to increase feed efficiency. Feed efficiency is affected by multiple factors such as basal metabolic rate, production level, absorbed profile of energy substrate, genetics, and gut microbial populations. The two factors investigated in this work were skeletal muscle metabolic rate and VFA dynamics because these factors are easily measurable, quantifiable, and have a shorter intervention interval (i.e., genetics can take years to alter). If skeletal muscle metabolic rate is measured from multiple muscle types and locations, then basal metabolic rate could be calculated allowing better understanding of individual animal maintenance requirements. Feed type, substrate source, rumen environment (e.g., pH), rumen fluid volume and flow, and microbial populations and attachment affect the fermentation dynamics within the rumen. The work described in this thesis focuses on investigating different aspects of feed efficiency.

The work described in Chapter 2 focuses on skeletal muscle metabolic activity, evaluating a strategy for quantifying metabolic activity and determine the correlation of this measurement with production parameters like growth and efficiency. The objective of this experiment was to determine the repeatability, practicality, and sensitivity of this assay in quantifying bovine skeletal muscle metabolic activity. The major conclusions from Chapter 2 are 1) the common cell culture assay was practical to conduct, 2) the assay could detect diet differences, and 3) the assay was sensitive enough to rank animals based on skeletal muscle metabolic activity. This assay has considerable potential to advance our understanding of individual animal maintenance requirements or stratify animals based on efficiency. Despite promising initial results, repeated assessment of the relationship between relative fluorescence and productive parameters like growth and efficiency are needed to confirm the usefulness of this assay.

Chapter 3 describes a study that measured the effects of substrate degradability on rumen fermentation characteristics. The objective of this work was to evaluate VFA, rumen fluid volume, passage rate, epithelium, and blood metabolite responses to different nutrient degradabilities. Accurately describing how substrate sources impact fermentation and epithelial gene expression is important to advancing our knowledge on how to feed animals more efficiently. This study demonstrated that nutrient degradability is a factor driving VFA dynamics and subsequent epithelial responses. The fiber sources used in this study generated greater shifts in fermentation than the starch sources. Additional work on starch sources differing in extent of degradation is needed to understand whether this relative importance is simply reflecting the relative importance of extent of ruminal degradation versus rate of degradation. The work also provides further evidence of the need for more holistic evaluation of how diets influence the fermentation environment of the rumen, including the epithelium.

Overall, the present work contributes to advancing our understanding of feed efficiency. One critical advancement was confirming a novel assay could be used to quantify skeletal muscle metabolic activity. Understanding metabolic rates in an inexpensive, sensitive, and labor-efficient way will improve the scientific community's knowledge of individual animal maintenance requirements to better formulate rations that are beneficial to the animal, maximize available resources, potentially mitigate harmful environmental effects, and are cost effective to the producer. The second advancement associated with this work was quantifying the importance of fiber sources (timothy hay, beet pulp) and starch sources (barley, corn) in regulating VFA concentrations, molar proportions, and fluxes; fluid volume and passage rate; epithelial gene expression; and blood metabolite concentrations. Understanding substrate source impacts on VFA dynamics allows nutritionists to be more specific when choosing feedstuffs. This specificity in

selection can help to potentially eliminating more expensive ingredients from a ration while maintaining or increasing production through enhanced feed efficiency and sustained rumen health.