Jersey Steer Ruminal Papillae Histology and Nutrigenomics

by

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Abstract

The transition from a high forage to a high concentrate diet is an important milestone for beef cattle moving from a stocker system to the feedlot. However, little is known about how this transition affects the rumen epithelial gene expression. This study assessed the effects of the transition from a high forage to a high concentrate diet as well as the transition from a high concentrate to a high forage diet on a variety of genes as well as ruminal papillae morphology in fistulated Jersey steers. In addition, the effects of using whole versus cracked corn on rumen papillae were ascertained by switching from whole corn to cracked corn 51 days after the start of the study. Jersey steers (n = 5) were fed either a high forage diet (100% forage) and transitioned to a high concentrate diet (20% forage and 80% grain) or a high concentrate diet that was transitioned to a high forage diet. Papillae from the rumen were collected for histology and RT-qPCR analysis. Histological analysis showed a relative increase in papillae length as concentrate was added to the diet in steers transitioning from a high forage to a high concentrate diet (P = 0.05). Genes related to cell membrane structure (*CLDN1, CLDN4, DSG1*), fatty acids metabolism (*CPT1A, ACADSB*), glycolysis (*PFKL*), ketogenesis (*HMGCL, HMGCS2, ACAT1*), lactate/pyruvate (*LDHA*), oxidative stress (*NQO1*), tissue growth (*AKT3, EGFR, EREG, IGFBP5, IRS1*), and urea cycle (*SLC14A1*) had a significant diet change × treatment interaction ($P < 0.05$). All of these changes can be considered indicators of rumen epithelial adaptation in response to changes in diet. In conclusion, these results

indicate changes in the composition of the diet can alter the expression of genes which function in rumen epithelial metabolism.

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List of Abbreviations

ACADSB	Acyl-CoA Dehydrogenase Short/Branched Chain
ACAT1	Acetyl-CoA Acetyltransferase 1
ADF	Acid Detergent Fiber
ADG	Average Daily Gain
AKT3	AKT Serine/Threonine Kinase 3
CLDN1	Claudin 1
CP	Crude Protein
CPT1A	Carnitine Palmitoyltransferase 1A
DM	Dry Matter
DMI	Dry Matter Intake
DSG1	Desmoglein 1
EGFR	Epidermal Growth Factor Receptor
ERBB4	Erb-b2 Receptor Tyrosine Kinase 4
EREG	Epiregulin
FA	Fatty Acid

FOM	Fermentable Organic Matter
H&E	Hematoxylin and Eosin
HMGCL	3-Hydroxymethyl-3-Methylglutaryl-CoA Lyase
HMGCS2	3-Hydroxy-3-Methylglutaryl-CoA Synthase 2
HVAC	Heating Ventilation and Air Conditioning
IGFBP1	Insulin Like Growth Factor Binding Protein 1
IGFBP5	Insulin Like Growth Factor Binding Protein 5
IMF	Intramuscular Fat
IRS1	Insulin Receptor Substrate 1
LCFA	Long Chained Fatty Acids
LDHA	Lactate Dehydrogenase A
MR	Milk Replacer
NDF	Neutral Detergent Fiber
NEFA	Non-esterified Fatty Acid
NQO1	NAD(P)H Dehydrogenase Quinone
PCR	Polymerase Chain Reaction

PFKL	Phosphofructokinase, Liver Type
PL	Papillae Length
PW	Papillae Width
RFI	Residual Feed Intake
RT-PCR	Real Time Polymerase Chain Reaction
SCFA	Short Chained Fatty Acid
SLC14A1	Solute Carrier Family 14 Member 1
TDN	Total Digestible Nutrients
TNFα	Tumor Necrosis Factor α
VFA	Volatile Fatty Acid

Chapter I

Literature Review

Background

Nutrigenomics studies the interplay of nutrition and genomics. The first portion of this field of study (i.e., nutrition), is an area of research that can lead to great financial savings. Feed costs are generally about 70% of the cost associated with raising livestock, so a large amount of research has been done on how to make animals more efficient in order to mitigate this large cost. The second part of nutrigenomics, genetics, is relatively a new field. The bovine genome was sequenced in 2009 by the Bovine Genome Sequencing Consortium and it has been published in the *Science Journal*, but there is still much to learn about individual genes' roles in a variety of processes, including nutrient metabolism. With the rumen comprising approximately 70% of an adult ruminant's gut space (Church *et al.*, 1988) and being responsible for up to 85% of the absorption of certain nutrients (Bannick *et al.*, 2008), the rumen epithelial transcriptome is a good target for nutrigenomic research. Better understanding of how expression of various genes in the rumen epithelium can be impacted by the alteration of nutrients provided can lead to improvements in productivity and feed efficiency of meat producing animals.

Rumen Epithelium

The rumen epithelium is divided into four layers: stratum basale, stratum spinosum, stratum granulosum, and stratum corneum (Stevens, 1969). The rumen is made up of stratified squamous epithelium, which is usually present in protective rather than absorptive tissues. Due to the fact that the epithelium in the rumen must act as a barrier between the rumen microbiome and the animal's circulatory and lymphatic systems, this unique architecture is important (Baldwin & Connor, 2017). The outermost layer, the stratum cornea, is likely for defense, forming a protective layer and having very few organelles. The stratum cornea has a higher number of cell layers when fed high concentrate diets compared to high forage diets (Gaebel *et al.*, 1987). In comparison, cells within the stratum basale, the layer closest to the basal lamina, are filled with fully functional organelles. These organelles, specifically the mitochondria, are the reason why the stratum basal contributes most to the papillae's metabolism. The stratum basale is the layer closest to blood supply while the stratum corneum lines the lumen (Stevens, 1969). The stratum granulosum has the most of tight junctions, which limit the passage of molecules and ions through the spaces between cells (Henrikson & Stacy, 1971) while the stratum basale has the most functional mitochondria (Stevens, 1969). The majority of Na^+- K^+ pumps in the rumen epithelium are found in the stratum basale, while the concentration of these pumps tails off towards the rumen which explains the higher density of mitochondria.

The most vital role of the rumen epithelium is the absorption of microbial fermentation products since it directly absorbs 50-85% of volatile fatty acids (VFAs). The rate of absorption of these nutrients is greatly affected by the size of the ruminal papillae, increasing with the increase in surface area (Bannink *et al.,* 2008; Melo *et al.,* 2013). Conversely, the different rumen epithelium layers can be affected by diet. For example, lambs fed milk and starter diet as opposed to just milk had increased cell layers in the stratum corneum, stratum granulosum and total epithelia (Sun *et al.,* 2018).

Rumen Development

Rumen development is a vital and dramatic process. With age, the rumen enlarges, changes its metabolic function, and increase the number of papillae (Hamada *et al.,* 1976). Ruminal digestive tract conformation starts out very similar to that of monogastrics, but by weaning, the rumen has gone from occupying 30% to 70% of gut space. The rumen is lined with finger-like projections called papillae. The papillaes' ability to absorb nutrients will depend on their length, width, and density (Lesmeister *et al.,* 2004). During nursing, milk bypasses the rumen through the esophageal groove, a muscular fold that connects the reticulum directly to the omasum, resulting in young ruminants often having underdeveloped papillae (Church, 1988).

As one of the main rumen fermentation products, VFAs, are a major source of energy for ruminants. When the animal starts to receive solid feed, it enters the rumen and stimulates microbial and papillae development, increasing the amount of VFAs (Nocek *et al.,* 1984).

Furthermore, it is well known that VFAs, the final products of rumen fermentation, also drive the establishment of the ruminal papillae development (Nocek *et al.*, 1984). Out of the three primary VFAs (butyrate, acetate, and propionate), butyrate has a particularly powerful effect on the rumen wall structure (Cavini *et al.*, 2015) and is supplemented in a variety of forms to promote rumen development (Gorka *et al.*, 2018). Specifically, butyrate helps with papillae development; most likely due to a decrease in apoptosis, or cell death (Mentschel *et al.*, 2001). Normal development of papillae also requires short-chain fatty acids (SCFAs) (Sander, 1959). Although initially it was thought that papillae development was caused by the mechanical presence of feed in the rumen, multiple studies have used non-feedstuffs to try to physically stimulate papillae growth to no avail (Warner, 1956; Smith, 1961; Tamate, 1962). However, when SCFA were infused into the rumen, papillae grew in length (Tamate, 1962). The papillae development ramps up when the animal starts to receive a solid diet, especially one that is starch-based (Suarez, 2006). Feed particle size also affects rumen papillae development. In general, a finer textured diet with small particle size, leads to more keratinization while a coarser diet decreases it (McGavin & Morrill, 1976). Age at weaning also appears to affect rumen development. The increase in ruminal SCFA, blood β-hydroxybutyrate, and fecal starch is greater in calves weaned at six weeks compared to those weaned at eight weeks (Eckert *et al.*, 2015), hence, management techniques could also anticipate these mentioned changes in rumen development.

Molecular Absorption in the Rumen (Rumen Wall Activity)

Ruminant animals are more efficient in utilizing feedstuffs that monogastrics (i.e., swine, poultry, and humans) are unable to digest. Microbes in the rumen possesses enzymes that allow them to break down structural carbohydrates, such as cellulose. Thus, digestion by these microbes allows ruminants to benefit from otherwise unusable feed sources. The rumen epithelium is a site of absorption of the nutrients that the microbes have broken down. However, the rumen epithelium of a calf is unable to utilize cellulose-rich feedstuffs at birth; maturation and viable rumen fermentation must occur in order for the mature rumen to properly function (Sander, 1959).

Rumen papillae morphology changes with type of diet

Rumen papillae morphology is key to the digestive physiology. More efficient cattle have been shown to have a thicker rumen epithelium compared to their less efficient counterparts (Lam *et al.,* 2017). This characteristic is pliable and has been shown to be affected by diet. Sheep given supplementation during the cold season had rumen papillae which were significantly wider and had more surface area than sheep which were not supplemented (Jing *et al.,* 2018). Furthermore, the thickness of stratum spinosum and stratum basale increased linearly in response to increasing neutral detergent fiber (NDF) to starch ratio (Ma *et al.,* 2017).

When transitioned from milk to a solid feed, ruminants do start to show papillae development (Zintan *et al.*, 1999). In milk replacer (MR), addition of acetate and propionate (Sakata, 1979), probiotics (Agazzi *et al.,* 2014), and prebiotics (Gorka *et al.,* 2011) has been shown to increase this development. One key requirement for rumen papillae growth is the intake of fermentable organic matter (FOM). As FOM increases so does papillae size (Dieho *et al.*, 2016). Forage in calves' first solid diet has been shown to increase average daily gain (ADG) and dry matter intake (DMI) (Thomas, 1982; Khan *et al.*, 2011; Castells *et al.*, 2013).

The effects of a VFA can vary drastically depending on an animal's nutritional status and the rate at which VFAs are consumed (Sakata, 1976, 1978). Throughout a ruminant animal's life, the types of feed ingested will affect rumen papillae differently. For instance, increasing the amount of concentrates in the diet increases the rumen papillae length and density due to the increase of VFAs (Stobo *et al.,* 1966), specifically propionate and butyrate (Baldwin *et al.,* 1998). Introduction of forage will cause papillae to be shorter (Castells *et al.,* 2013). Even early on, cattle fed high concentrate diets develop longer papillae which result in better beef quality (Reddy *et al.*, 2017). In addition, calves whose diet were supplemented with 5% calcium propionate showed greater papillae length (Zhang *et al.,* 2018).

Corn Processing

The type and extent of corn processing changes how the feed is digested by the rumen. The less digestible a whole grain is, the more beneficial processing can be. The less starch that escapes the rumen, the more there is available to be utilized by the animal (Theuer, 1984). Even with minimal processing, such as cracking whole corn, final weight, daily gain, and dry matter intake increase (Turgeon, 1983). Whole corn is less ruminally-degradable than ground corn, however acetic acid production and pH remain similar even after processing (Landau *et al.,* 1992). Smaller particle sized corn increase organic matter digestibility (Callison *et al.,* 2001). When already processed, it has been shown that particle size does not affect average daily gain or other growth parameters (Bengochea *et al.,* 2005; Siverson *et al.,* 2014). Additionally, sheep fed a diet with a high amylose/amylopectin ratio early in life have been shown to have a better rumen epithelium morphology (Zhao *et al.,* 2017).

Ketogenesis

Ketosis occurs when too many ketone bodies accumulate in the blood and lower the rumen's pH to unhealthy levels. The main cause of ketosis is low intake, usually to the extent of the body being in a starvation state. Starvation causes this because the liver increases output of ketone bodies due to hormonal (i.e. insulin and glucagon) signals. This causes fat mobilization which in turn removes more VFAs (Brockman, 1979). Metabolism of VFAs leads to the formation of ketone bodies via ketogenesis primarily in

the liver and rumen (Heitmann, 1987). During ketogenesis, VFAs are converted into acetone, acetoacetate, or beta-hydroxybutyrate (βHBA). Ketogenic capacity of the rumen in neonates is virtually nonexistent (Warner, 1956). Although ketogenesis is an integral part of ruminant digestion, too much buildup of ketone bodies can cause problems such as ruminal acidosis or ketosis. Lactating cows and ewes pregnant with multiples are especially at high risk due to the energy demands of their physical states (Reid, 1968). Ketosis, which occurs in the rumen epithelium, can reduce these cells' ability to absorb nutrients (Leighton *et al.,* 1983). The rate at which ruminal ketogenesis occurs may be increased by the addition of glucose (Beck *et al.,* 1984).

Rumen Gene Expression

Despite feed being the costliest factor of cattle production, little is known about the effects of feeding on the rumen transcriptome. A connection between an animal's intake, weight gain, and their interaction has been found in relation to genes related to immunity (Kern *et al.,* 2016).

Cell Membrane Structure

Cell membrane structure and its integrity are fundamental for cell survival. Genes associated with membrane structure include Claudin-1 (*CLDN1*), Claudin-4 (*CLDN4*), and Desmoglein 1 (*DSG1*). CLDNs are associated with endothelial tight junction components (Collins *et al.,* 2006; Gunzel and Yu, 2013). Claudins are primarily expressed in the cells of the stratum granulosum (Graham & Simmons, 2005). In

lactating Holstein cows, both *CLDN1* and *CLDN4* expression were upregulated in rumen epithelium with the lower pH of induced subacute ruminal acidosis (SARA). However, no mechanism for this upregulation has been found (McCann *et al.*, 2016). Claudin-4 expression was significantly increased in the intestinal mucosa lesion of mice (Tamura *et al.*, 2008). *DSG1* is associated with cellular transport and structure through dynamic changes in ion binding (Wang *et al.*, 2017). *DSG1* is highly expressed in stratified squamous epithelium, especially the stratum granulosum (Green *et al.*, 2007). In the rumen epithelium of cattle fed a high grain diet, *DSG1* was down regulated while expression was highest in those fed a high forage diet. This down regulation may be due to the disruption of tight junctions and it is hypothesized either increased permeability or paracellular transport may be one of the mechanisms by which SCFA are cleared from the rumen in response to the increased organic acid load imposed on the ruminal environment during SARA (Steele *et al.*, 2011). Similarly, *DSG1* expression was also upregulated in the rumen epithelium of cattle recovering from an acidotic bout (McCann *et al.*, 2016).

Fatty Acids

Lipids are hydrolyzed into fatty acids in the rumen and later absorbed in the small intestine (Harfoot, 1978). The extent of hydrolysis depends upon rumen pH and may therefore vary between diets (Gerson *et al.*, 1985). Fatty acids are an important source of energy in ruminants. Two examples of the genes linked to fatty acid regulation include

Acyl-CoA dehydrogenase short/branched chain (*ACADSB*), and Carnitine palmitoyl-transferase 1A (*CPT1A*). *ACADSB* is associated with short/branched-chain FA metabolism (Rozen *et al.,* 1994); more specifically the first step of the mitochondrial β-oxidation reaction (Ensenauer *et al*., 2005). However, it has been shown to be differentially expressed in bovine mammary epithelium (Jiang *et al.,* 2018). *CPT1A* catalyzes the entry of LCFA into mitochondria and the first step of mitochondrial β-oxidation of LCFA (Zammit, 1984). *CPT1A* expression in the rumen epithelium of calves was not affected by varying milk replacer treatments, however expression was down-regulated with time (Naeem *et al.,* 2012). In rat liver, *CPT1A* transcription has been shown to increase with increasing levels of glucose, but its translation was not affected (Serviddio *et al.,* 2011) with a small increase in NEFA concentrations (i.e., up to 1.2 mmol/L) transcription and translation of *CPT1A* mRNA was increased. Although, from 1.2 to 4.8 mmol/L both decreased. This higher concentration of NEFA also reduced fatty acid oxidation (Xu *et al.,* 2011). *CPT1A* mRNA expression in the liver of dairy cows has also been shown to be associated with decreased incidence of metabolic and reproductive disorders (Graber *et al.,* 2012). In sheep, *CPT1A* expression was found to be higher in the mucosal epithelium of the large intestine than the duodenum (Chao *et al.,* 2017).

Glycolysis

Glycolysis is the process in which glucose is broken down for energy. This produces Adenosine Triphosphate (ATP) and is the primary energy source for animal cells.

Phosphofructokinase, liver type (*PFKL*) is one gene associated with this process. *PFKL* catalyzes the phosphorylation of fructose 6-phosphate to fructose 1, 6-bisphosphate. This irreversible reaction serves as the major rate-limiting step of glycolysis (Graham *et al.,* 2015). Mice with overexpression of *PFKL* show shifts in glucose metabolism and a decrease in the glucose-induced insulin response (Knobler *et al.,* 1997). In humans, *PFKL* levels have been shown to be significantly affected by low fat, but not low carbohydrate diets (Seip *et al.,* 2008).

Ketogenesis

Ketogenesis is the formation of ketone bodies, heavily associated with fat mobilization and increased VFA removal (Brockman *et al.,* 1979). Genes associated with ketogenesis include acetyl-CoA acetyltransferase 1 (*ACAT1*), 3-hydroxymethyl-3-methylglutaryl-CoA lyase (*HMGCL*) and 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*HMGCS2*). *ACAT1* encodes a mitochondrially localized enzyme that catalyzes the reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA (Chang, 1993). This is the first step in ketogenesis (Lane *et al.,* 2002). Bulls fed a high concentrate diet increased absorption of acetic acid due to greater *ACAT1* activity in rumen epithelium (Harmon *et al.,* 1991). In Angus × Hereford steers, *ACAT1* trended towards increased expression with increased Residual Feed Intake (RFI) (Kern *et al.,* 2017). *ACAT1* decreases in expression late in fattening (Lee *et al.,* 2007).

HMGCL contributes to ketogenesis by converting HMG-CoA to acetoacetate (Lane *et al.,* 2002). *HMGCL* has been shown to increase with larger amounts of grain in the rumen epithelium of cattle which experience a decrease in rumen pH with the increase of grain (Zhao *et al.,* 2017). Laarman *et al*. 2012 found *HMGCL* to not be associated with butyrate production, however Wang *et al.* 2016 found *HMGCL* and *HMGCS2* to be associated with changes in VFA levels, particularly butyrate. In the rumen of sheep, both *HMGCL* and *HMGCS2* were both shown to be upregulated in lambs fed a starter diet in comparison to those that nursed (Wang *et al.,* 2016). *HMGCS2* plays a central role in coordinating ruminal ketogenic flux (Naeem *et al.,* 2012).

Lactate/Pyruvate metabolism

Lactate dehydrogenase A (*LDHA*) catalyzes the conversion of L-lactate and NAD to pyruvate and NADH in the final step of anaerobic glycolysis (Valvona *et al.,* 2016). *LDHA* levels decreased in calves fed an enhanced plane of nutrition, possibly leading to greater utilization of circulating amino acids for gluconeogenesis by ruminal tissue (Naeem *et al.,* 2014).

Oxidative Stress

Oxidative stress is caused by the overabundance of reactive oxygen species (ROS) and antioxidants. This imbalance can lead to cellular damage. In dairy cattle it has been shown that cows with higher BHBA and NEFA showed higher reactive oxygen metabolites (ROM) and lower levels of antioxidants. Additionally, cows that had higher

body condition score (BCS) and greater BCS losses during the peripartum period were more sensitive to oxidative stress (Bernabucci *et al.*, 2005). NADPH quinone dehydrogenase 1 (*NQO1*) protects cells from damage caused by oxidative stress by acting as a substrate for the two-electron transferring flavoenzymes (Sarlauskas *et al.*, 2004). In the rumen papillae of Angus × Hereford steers, *NQO1* had increased expression in low residual feed intake (RFI) individuals (Kern *et al.*, 2017), hence, *NQO1* could be considered as a marker for feed efficiency. Additionally, in cattle fed grain to the point of SARA, *NQO1* expression was downregulated (Abaker *et al.*, 2017).

Tissue Growth/Structure

AKT serine/threonine kinase 3 (*AKT3*) encodes for RAC-gamma serine/threonine-protein kinase. AKT kinases regulate cell signaling in response to insulin and growth factors; *AKT3* seems particularly relevant to the brain (Nakatani *et al.*, 1999). *AKT3*, via cell proliferation, can coordinate an increase in organ weight in mice (Dummler *et al.*, 2006). However, it has been found that calves with enhanced early plane of nutrition initially express lower amounts of *AKT3* before it increased at the end of the mild-fed period (Naeem *et al.*, 2014).

Epidermal growth factor receptor (*EGFR*) is a glycoprotein that binds to epidermal growth factor leading to cell proliferation (Yarden & Schlessinger, 1987). *EGFR* had been found to be a focal regulatory gene in the adaptive response of rumen papillae in dairy cattle during early lactation (Steele *et al.*, 2015). Epiregulin (*EREG*) acts as a ligand

for both epidermal growth factor receptor (*EGFR*) and the erb-b2 receptor tyrosine kinase 4 (*ERBB4*). It is believed to be involved in a variety of processes including inflammation, wound healing, and cell proliferation (Toyoda *et al.,* 1997). In rumen papillae, both *EREG* and *EGFR* expression increased as Holstein cows progressed from day 10 to day 28 in milk (Minuti *et al.,* 2015).

Insulin Growth Factor Binding Protein 5 (*IGFBP5*) is a factor in muscle cell development (Zhang *et al.,* 2017). It is also associated with cellular apoptosis, proliferation, and differentiation specifically in mammary tissue (Mohapatra *et al.,* 2015). In porcine smooth muscle tissue, *IGFBP-5* has been shown to potentiate IGF-I activity, encouraging cellular proliferation. *IGFBP-5* may have an anti-apoptotic role in the survival of these cells during differentiation as it prevented apoptosis induced by TNFα (Firth *et al.,* 2002). Expression of *IGFBP-5* mRNA has been shown to be upregulated in the rumen papillae of sheep supplemented with a higher plane of nutrition and in cattle transitioning to a high grain diet (Jing *et al.,* 2018; Lei *et al.* 2018; Steele *et al.,* 2011; Steele *et al.,* 2012).

Insulin Receptor Substrate 1 (*IRS1*) plays a role in signaling from insulin and Insulin Growth Factor Binding Protein 1 (*IGFBP1*) receptors to various intracellular pathways. *IRS1* phosphorylation begins a cascade of events that leads to glucose uptake. This signaling and subsequent uptake of glucose leads to tissue proliferation (Higashi *et al.,* 2012). In the rumen epithelium of multiparous Holstein cows, *IRS1* has been shown to

increase with a transition to a higher energy, lower straw diet. The authors surmise that this may increase insulin sensitivity (Minuti *et al.*, 2015).

Urea Cycle

Solute carrier family 14 member 1 (*SLC14A1*) mediates the basolateral cell membrane transport of urea across the rumen epithelium (Abdoun *et al.*, 2010). In the rumen, urea is turned into ammonia which is used by rumen microbes as a nitrogen source. As these microbes turn over and are digested in the intestine, this nitrogen returns to the animal as microbial cell protein, an important energy source (Abdoun, Stumpff, & Martens, 2006). Furthermore, *SLC14A1* has been shown to be upregulated by solid feed intake; this may be because of urea recycling (Berends *et al.*, 2014). *SLC14A1* is expressed and may transport urea into the rumen (Stewart *et al.*, 2005). In the rumen epithelium of sheep, it has been shown to not be differentially expressed (Xiang *et al.*, 2016). It has been demonstrated that *SLC14A1* expression is upregulated in the rumen epithelium of sheep fed a high concentrate diet (Lu *et al.*, 2014).

Chapter II

Gene Expression Response of the Rumen Epithelium of Jersey Steers During Transition from Different Diets

Introduction

The rumen must go through a period of adjustment when cattle are switched from pasture to feedlot and therefore from a high forage to a high grain diet. Understanding the changes the rumen undergoes during this transition is necessary due to its responsibility for the majority of nutrient absorption. The impact of high concentrate diets on rumen epithelium have been previously studied, showing that the diet leads to an increase in papillae size and cell layers (Stobo *et al.,* 1996; Warner *et al.,* 1956).

The rumen changes in response to various diets. Identification of gene responses driving these changes may enhance our ability to formulate diets and better transition cattle from the pasture to the feedlot. A contingent of studies have shown the effects of different types of diets on aspects of metabolism including cell membrane structure (Collins *et al.,* 2006; McCann *et al.,* 2016; Steele *et al.,* 2011), cytokines (Kern *et al.,* 2017), fatty acids (Jian *et al.,* 2018; Xu *et al.,* 2011), glycolysis (Knobler *et al.,* 1997), ketogenesis (Lane *et al.,* 2002), lactate/pyruvate metabolism (Naeem *et al.,* 2014), oxidative stress (Abaker *et al.,* 2017), tissue growth (Steele *et al.,* 2015), and the urea cycle (Stewart *et al.,* 2005). On the basis of previous studies focused on gene expression and the ruminal epithelium, we looked at gene expression patterns of genes related to these various metabolic processes.

To better understand ruminal cell metabolism and proliferation, genes across wider number of processes should be studied. We have used RT-PCR as a tool to study the effects of varying diets on targeted gene expression. Data were used to uncover possible metabolic processes which are influenced by transition to a different diet.

Materials and Methods

Animals and Sampling

Five, two-year old, fistulated Jersey steers from Auburn University's College of Veterinary Medicine were used in this study. Animals were housed in a barn equipped with a heating ventilation and air conditioning (HVAC) controlled environment system. Animals were located individually in 10 × 10 feet pens, where they were able to have physical contact with their neighbors. Pens were equipped with individual water troughs and feed bunks. Animals had *ad libitum* access to water and approximately 20 pounds per day of ration was provided at 0700 and 1500 hours.

Dietary treatments were based on the transition from a high fiber diet to a high concentrate diet (Treatment A) and from a high concentrate diet to a high fiber diet (Treatment B). There were three animals that received Treatment A and two animals assigned to Treatment B. Days on treatment for both groups were 122 days in total. The study began with a 14-day feeding period to allow the steers to adapt to their treatment diets.

Jersey steers assigned to Treatment A started receiving a diet consisting of 100 % forage and 0 % grain (F:G = 100:0). After 21 days on this diet, it was changed to F:G = 80:20

(Days 22 to 58 on treatment) using whole corn. From day 59 to 74 steers received a F:G = 80:20 but this time diet was prepared with cracked corn. Next, steers received a diet F:G = 60:40 (Days 75 to 90 on treatment), followed by a diet F:G = 40:60 (Days 91 to 106 on treatment) and the study finished with a diet F:G = 20:80 (Days 107 to 122 on treatment). Animals that belonged to Treatment B had a period of adaptation of 14 days to a high grain diet before the beginning of the administration of the dietary treatments. For this pre-conditioning of the animals to a high grain diet, the addition of whole corn grains to the diet was gradual until it reached the amount of grain required for the dietary treatment that they received for the first 21 days on treatment (F:G = 20:80). The following 22 to 58 days on treatment, they received a whole corn based diet (F:G = 40:60). From 59 to 74 days on treatment, they received a cracked corn based diet (F:G = 40:60). From 75 to 90 days on treatment, diet was F:G = 60:40, from 91 to 106 days on treatment, diet was F:G = 80:20 and from 107 to 122 days on treatment, the diet was 100% forage.

Diets were formulated to meet animal's nutritional needs based on the NRC recommendations for beef cattle. The concentrate mix, in an as-fed basis, consisted of 14.18% distillers grains, 49.64% corn, 18.44% dry cottonseed hulls, 10.64% dry soybean hulls, 4.96% molasses, 1.42% A&M 8% phosphorous, and 0.71% 38% limestone. The forage used was a bermudagrass hay. On an as-fed basis the bermudagrass hay had 92.63% DM, 9.83% CP, 32.56% ADF, 61.70% NDF, 4.63% lignin, and 55.96% TDN. All cattle had free access to a trace mineral salt block, which contains 96% NaCl, 2,400

ppm Mn, 2,400 ppm Fe, 260 ppm Cu, 320 ppm Zn, 70 ppm I and, 40 ppm Co (American Stockman® Big 6 Trace Mineral Block).

Rumen epithelium biopsies were performed before changing diets, at 1, 23, 52 and 76 days on treatment, at 91 days on treatment (last day that coincidentally both treatments receive the same diet) and at 107 days on treatment and, at 122 days on treatment (end of study).

Rumen epithelium biopsy was performed under field conditions at each animal's individual pen prior to the morning feeding. Instruments for the surgical procedures were sterilized by autoclaving. Surgical scissors were placed in chlorhexidine diacetate disinfectant solution (Nolvasan, Zoetis Animal Health, Parsippany, New Jersey) prior to ruminal biopsy and rinsed with sterile saline solution before harvesting the tissue. Prior to biopsy the rumen was partially emptied. Rumen contents were kept in a covered bucket and were returned to the rumen immediately after the biopsy. Once partially empty, the ventral sac area of the rumen was retracted up to the cannula where papillae were excised using surgical scissors. Feed particles were rinsed away with water prior to harvesting epithelium. Approximately 0.5 grams of papillae (wet weight) and an additional five individual papillae from each steer at the specified times were biopsied. Furthermore, prior to biopsy, samples of rumen fluid were collected in polypropylene tubes and the electrode of a pH meter was submerged on the tube to measure pH immediately after collection.

Rumen Papillae Histology

The rumen papillae histological analysis followed a protocol previously described (Ragionieri *et al.* 2016). Briefly, three papillae samples from each animal at each time point were immersed in 10% neutral buffered formaldehyde for 5 d, after being fixed with pins on cork discs to prevent shrinkage. Then the samples were dehydrated and processed using a Thermo Scientific Spin Tissue Processor Microm STP-120 according to manufacturer instructions. Following fixation, the papillae were individually imbedded in paraffin wax. Histological sections (4 μm thick) were then stained with hematoxylin and eosin (H&E) for morphometric analysis under a light microscope (Nikon Eclipse TS100 microscope, TS 100LED-F-MV) equipped with a digital camera (Nikon Digital Sight U3 camera, DS-U3). Slides were then read in a random order using Nikon Computer Software. The papillae were measured length wise and width wise at the most medial point. Papillae length (PL) (distance between the base and the tip of the papillae) and width (PW) (at the middle of the papillae) were measured.

RNA Extraction

Using a beadbeater, 50 mg of rumen epithelium from each animal at each collection date was homogenized for 30 seconds with 1 mL of qiazol solution (Qiagen miRNeasy Mini Kit; Cat. #: 217004), cooling on ice for one minute and then homogenizing again for another 30 seconds. The samples were then centrifuged for 10 minutes at 12,000 rpm at 4ºC to remove insoluble material. The homogenized portion was transferred to a new

tube and allowed to sit at room temperature for 5 minutes before being put on ice. Then, 200 µl of chloroform was added and each sample and tubes were shook for 15 seconds to mix. The samples then sat for 3 minutes at room temperature. Then they were centrifuged for 15 minutes at 12,000 rpm at 4ºC. The upper phase containing the RNA was transferred to a new tube and 750 µl of 100% ethanol was mixed in. To clean the extracted total RNA, 700 µl of the sample was pipetted into a miRNeasy spin column (Qiagen miRNeasy Mini Kit; Cat. #: 217004). This was then centrifuged for 12,000 rpm for 15 seconds. These three steps were repeated until the totality of the sample went through the spin column. Then 350 µl of RWT buffer was added to each column and centrifuged again at 12,000 rpm for 15 seconds. Following, 80 µl of DNase I digestion mix (Qiagen, Hilden, Germany) was added to each column and was incubated for 15 minutes. An additional 350 µl of RWT buffer was added to each column and centrifuged again at 12,000 rpm for 15 seconds. Next, 500 µL RPE buffer was added to the miRNeasy mini spin column which was centrifuged for 15 seconds and after discarding the flow through, a final centrifugation of 2 minutes was performed to completely dry the column. Finally, 50 µL of RNase free water was placed directly onto the mini spin column membrane and centrifuged for 1 minute at 12,000 rpm to elute RNA. Concentration of extracted RNA was measured using a Nanodrop OneC instrument.

Primer Design

The cDNA sequence for each gene of interest was found at University of California-Santa Cruz's Genome Browser (https://genome.ucsc.edu/) or National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/). This sequence was inputted into Primer Express 3.0.1 software (ABI). The default settings (TaqMan® MGB quantification) was used, except the minimum amplicon size was adjusted to 100 base pairs. The designed primer sequences were then blasted using NCBI Nucleotide Blast and ordered from Integrated DNA Technologies (https://www.idtdna.com). Primer information can be found in Additional Table 2.

cDNA Synthesis

First the total RNA was diluted to a concentration of 100 ng/µL. Then Master Mix 1 (MM1) was prepared by mixing 9 µL of RNase free water to 1 µL of Random Primers (Roche Diagnostics, Indianapolis, IN). Then 1 µL of 100 ng total RNA was added. Next, the mixture was incubated at 65°C for 5 minutes. After this first incubation, the samples were kept on ice for 3 minutes. For each sample, Master Mix 2 (MM2) was prepared by mixing 1.625 µL RNase free water, 4 µL 5X First-Strand Buffer,1 µL Oligo dT18, 2 µL 10 mM dNTP mix (10 mM), 0.25 µL of Revert aid (200U/µl), and 0.125 µL of Rnase inhibitor (20U/µl). Finally, MM2 was added to MM1+RNA (final volume of 20 uL and multiplied based on number of genes analyzed), then incubated using the following temporal profile: 25°C for 5 min, 42°C for 60 min and 70°C for 5 min

followed by 4°C. Standard curve consisted on 7 points. An aliquot of cDNA from each sample was pooled. This pooled sample was then diluted to a 1:2 ratio with molecular grade water for the first point. The subsequent points were diluted down to a 1:4 ratio.

Preliminary Primer Testing

In a PCR tube, 8 µL of pooled cDNA, 10 µL of Perfecta SYBR Green, 1 µL of Forward primer, and 1 µL of Reverse primer for each tested gene was added. The samples were placed in an Eppendorf nexus gradient thermocycler for 2 min at 50°C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 minute at 60 °C (denaturation). Five µL of the PCR product were transferred into a new 0.2 mL PCR tube for agarose gel electrophoresis analysis and 2 µL of loading dye were added and vortexed together. The ladder was prepared by vortexing together 0.6 µL of ladder (25 bp, from Invitrogen) with 2 µL of loading dye. Three grams of agarose where dissolved in 150 mL of 1X TAE buffer. Two µL of SYBR Safe were added before loaded in the agarose gel apparatus. The ladder was added to each row and the samples were also added to their corresponding well. The gel ran at 80mV until the samples had made it ¾ of the way across the gel. The gel was then analyzed in the Bio-Rad Chemi Doc apparatus using Image Lab software. PCR products from tested primers with a single, clear band at 100 bp were deemed acceptable.

The protocol suggested by the manufacturer (QIAGEN) for the kit (QIAquick® PCR Purification Kit) was used to clean PCR product before sending for sequencing. Samples were sent for sequencing to the University of Illinois Core Sequencing facility and the

sequencing results were blast in NCBI. Only sequencing results with NCBI blast that matched the primers blast were used (Additional Table 3). Genes selected for transcript profiling in the present study were grouped as follows: cellular membrane structure, claudin 1 (*CLDN 1*), claudin 4 (*CLDN4*), and desmoglein 1 (*DSG1*); fatty acid metabolism, acyl-CoA dehydrogenase short/branched chain (*ACADSB*), and carnitine palmitoyltransferase 1A (*CPT1A*); glycolysis, phosphofructyokinase, liver type (*PFKL*); ketogenesis, 3-hydroxymethyl-3-methylglutaryl-CoA lyase (*HMGCL*), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*HMGCS2*), acetyl-CoA acetyltransferase 1 (*ACAT1*); lactate/pyruvate, lactate dehydrogenase A (*LDHA*); oxidative stress NAD(P)H dehydrogenase quinone (*NQO1*); tissue growth and structure, AKT serine/threonine kinase 3 (*AKT3*), epidermal growth factor receptor (*EGFR*), epiregulin (*EREG*) and, insulin like growth factor binding protein 5 (*IGFBP5*); insulin receptor substrate 1 (*IRS1*); and the urea cycle, solute carrier family 14 member 1 (*SLC14A1*) (Supplemental Tables 1-3).

RT-PCR

Four µL diluted cDNA sample, negative controls, and standard curve were pipetted in their respective wells in a MicroAmp™ Optical 96-well reaction plate in triplicate. Then, 12 µL of SYBR Green Master Mix was placed in each well. The PCR reaction was performed in an ABI Prism 7500 HT SDS instrument with the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. The RT-

qPCR data was then analyzed using the 7500 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems).

Statistical Analysis

Quantitative PCR data was analyzed using the MIXED procedure of SAS (SAS 9.4 Institute, Cary, NC, USA). Before statistical analysis, normalized RT-qPCR data (using the geometric mean of Ubiquitously expressed prefoldin like chaperone (*UXT*), and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which are internal control genes). Fixed effects in the statistical model for each variable analyzed (i.e., genes, or animal performance) included dietary treatment (F:G or G:F), dietary change (100:0, 80:20, 60:40, etc.), and interactions of second and third order. Gene expression data analysis included a repeated-measures statement with an autoregressive covariate structure. Animal performance (steer body weight and rumen pH) was also analyzed using the MIXED procedure of SAS. The random effect in all models was steer within treatment. Significant differences were declared at $P \leq 0.05$ and tendencies between $0.06 \leq P \geq 0.1$.

Results

Animal Performance

There was significant diet change × treatment interaction for body weight ($P < 0.01$) and a diet change effect ($P < 0.01$), but there was not a treatment effect ($P = 0.59$). The animals from treatment A lost body weight during the first 51 days on treatment before

gaining weight throughout the rest of the study (Figure 1). In addition, there was a tendency for significant diet change × treatment interaction for ruminal pH ($P = 0.07$). and a diet change effect ($P < 0.01$) with no significant treatment effect ($P = 0.14$) in pH was seen with all steers. Throughout the study, rumen fluid pH was maintained within 6.6 and 7.3 (Figure 2). Interestingly, both treatments had a significant increase in body weight ($P < 0.01$) when whole corn was replaced with cracked corn on the diet (Figure 1).

Histology

There were significant diet change × treatment interaction ($P = 0.05$), but not treatment ($P < 0.11$) or diet change ($P < 0.21$) effects for rumen papillae length. Rumen papillae width was not significantly affected by diet change ($P < 0.26$), treatment ($P < 0.69$), or diet change × treatment interaction ($P < 0.37$) (Figure 3).

Gene expression

Additional Table 1 depicted the qPCR performance of all genes analyzed. Relative mRNA abundance which is the individual proportion of mRNA present in the rumen tissue of each gene was calculated. From all genes under study, *LDHA* had the greatest relative mRNA abundance (36%) in the rumen papillae and *IRS1* presented the lowest relative mRNA abundance (0.01%) throughout the study (Figure 4).

Cell Membrane Structure

There was a significant treatment × diet change interaction for *CLDN1* ($P < 0.01$) and a diet change effect ($P < 0.01$) (Table 1). In treatment A steers, *CLDN1* expression was inconsistent until the animals switched to a 60:40 diet, where its expression increased with the increased amount of grain in the subsequent diets. In treatment B steers, *CLDN1* expression decreased as the proportion of grain decreased, although a ~1-fold increase in *CLDN1* expression was observed when grain was completely removed from the diet (100:0). Additionally, *CLDN1* expression was significantly different between 80:20 C diet for treatment B and 40:60 C diet for treatment A. There was a tendency for treatment × diet change interaction for *CLDN4* ($P = 0.06$) and a diet change effect ($P < 0.01$). (Figure 5).

There was a significant treatment × diet change interaction for *DSG1* ($P < 0.01$) and a diet change effect ($P < 0.01$). In treatment A steers, there was a sharp decrease in *DSG1* expression when grain was initially added. In treatment B steers, *DSG1* expression remained stable throughout the study (Figure 5). Although, relative mRNA abundance of *DSG1* was only 0.09% as compared to *CLDN1* which had a 2.68%.

Fatty Acid Metabolism

There was a significant treatment × diet change interaction for *ACADSB* ($P < 0.01$) and a diet change effect ($P < 0.01$) (Table 1). In treatment A steers, *ACADSB* expression was inconsistent throughout the study but, this changes in gene expression were statistically

significant ($P < 0.05$). However, there was a significant increase when the steers were switched from whole to cracked corn and a more than 1-fold increase in *ACADSB* expression between the changes from a 60:40 diet to a 20:80 diet (Figure 6). In treatment B steers, there was consistency throughout the study for *ACADSB* expression.

There was a tendency for a treatment × diet change interaction for *CPT1A* ($P = 0.07$) and a diet change effect ($P < 0.01$) (Table 1). In treatment A steers, *CPT1A* had a significant decrease in expression for the first 21 days of the study and, *CPT1A* significantly increased with the change to a 20:80 diet. Steers on treatment B showed a similar pattern. There was a difference in *CPT1A* expression between treatment A and B at baseline (Figure 6).

Glycolysis

There was a significant treatment × diet change interaction for *PFKL* ($P < 0.01$) and a diet change effect ($P < 0.01$) (Table 1). In treatment B steers, *PFKL* expression significantly increased between the change from a 40:60 diet to a 60:40 diet, at which point expression decreased when under a 80:20 diet. However, *PFKL* expression did not present significant chances for treatment A steers. There was a difference in *PFKL* expression between treatment A and B after being both treatment under a 60:40 diet for 16 days (Figure 7).

Ketogenesis

There was a significant treatment × diet change interaction for *ACAT1* ($P < 0.01$) and a diet change effect ($P < 0.01$) (Figure 9). Treatment A had an increase in *ACAT1* expression after being under 100:0 diet for 21 days and when switched from a whole to cracked corn diet. *ACAT1* expression decreased in treatment A after 30 days under a 80:20 diet. Treatment B presented a relatively constant expression level until the switch to the 100% forage diet when *ACAT1* expression increased significantly. Both treatments had increased expression during the last 16 days of the study.

There was a significant treatment × diet change interaction for *HMGCL* ($P < 0.01$) and a diet change effect ($P < 0.01$) (Table 1). In treatment A steers, *HMGCL* expression increase significantly after 21 days under 100:0 diet and, at the end of the study, when steers from treatment A where under a 20:80 diet for 16 days. In treatment B steers, *HMGCL* expression increased during the first 21 days when on the 20:80 whole corn-based diet and decrease with 20% removal of corn from the 20:80 diet, then *HMGCL* expression stayed steady for the remaining of the study (Figure 9).

There was a significant treatment × diet change interaction for *HMGCS2* ($P < 0.01$) and a diet change effect ($P < 0.01$) (Figure 9). In treatment A steers, *HMGCS2* expression increased during the first 21 days under 100% forage diet. Additionally, there was also a large uptick in *HMGCS2* expression after the switch from whole to cracked corn on the diet. Although, the most important change in *HMGCS2* expression was during the last 16

days of the study, when under a 20:80 diet, where it had a significant up-regulation. In treatment B steers, *HMGCS2* presented an increase in its expression level during the 21 days under a 20:80 diet and, it remained relatively steady throughout the rest of the study.

Pyruvate/lactate metabolism

There was a significant treatment × diet change interaction for *LDHA* ($P < 0.01$), a diet change effect ($P < 0.01$) and a treatment effect ($P = 0.01$) (Table 1). In treatment A steers, *LDHA* expression increased with the addition of grain until switched from whole to cracked corn-based diet at which point expression decreased with the addition of grain. In treatment B steers, *LDHA* expression had a significant increase during the 21 days under a 20:80 diet, switching to a decrease in expression when a 40:60 diet was administered for a period of 30 days. There was a difference in *LDHA* expression between treatment A and B after being both treatment under a 60:40 diet for 16 days (Figure10).

Oxidative Stress

There was a significant treatment × diet change interaction ($P < 0.01$), diet change effect ($P < 0.01$), and treatment effect ($P = 0.01$) for *NQO1*. In treatment A steers, *NQO1* expression presented a significant increased when switching the diet from whole to cracked corn, and there was another significant increment in *NQO1* expression during the last 16 days of the study. In treatment B steers, *NQO1* expression remained relatively

steady. Finally, there was a significant difference in *NQO1* expression between treatments at the time of the second, fourth and last diet change (Figure 11).

Tissue Growth/Development

There was a significant treatment × diet change interaction for *AKT3* ($P < 0.01$), a diet change effect ($P < 0.01$) and a treatment effect ($P = 0.04$). In treatment A steers, there was a significant decrease of *AKT3* expression between the change from a 60:40 diet to a 40:60 diet and there was a significant increase after receiving the 20:80 cracked corn-based diet for 16 days. In treatment B steers, *AKT3* expression was inconsistent with a significant decrease between 20:80 and 40:60 diet change and between 80:20 and 100:0 diet. *AKT3* had also an increment in its expression level between 60:40 and 80:20 diet change. There was a significant difference in *AKT3* expression between treatments at the time of the fifth diet change (Figure 12).

There was a significant treatment × diet change interaction for *EGFR* ($P < 0.01$) and a diet change effect ($P < 0.01$) (Table 1). In treatment A steers, *EGFR* expression was slightly activated after 21 days under 100% forage base diet and it experienced also activation during the 16 days under a 20:80 diet. However, there was a decrease in *EGFR* expression when under a 60:40 diet for 16 days. In treatment B steers, *EGFR* expression remained constant throughout the study. However, there was a significant difference in *EGFR* expression between treatments at the time of the fourth diet change (Figure 12).

There was a significant treatment × diet change interaction for *EREG* ($P < 0.01$) with no treatment or diet change effect ($P > 0.05$). For both treatments, *EREG* expression was inconsistent (Figure 12).

There was a significant treatment × diet change interaction for *IGFBP5* ($P < 0.01$) and a diet change effect ($P < 0.01$) (Table 1). In treatment A steers, *IGFBP5* expression generally increased with the addition of grain in the diet, especially during the last 16 days of study when under a 20:80 diet. In treatment B steers, *IGFBP5* expression did not have a clear pattern with diet change (Figure 12).

There was a significant treatment × diet change interaction for *IRS1* ($P < 0.01$) and a diet change effect ($P < 0.01$) (Figure 8). In treatment A steers, expression remained relatively steady with no statistical significant differences due to diet change. In treatment B steers, *IRS1* expression had an important increment from baseline to 20:80 and a subsequent significant decrease in expression from 20:80 to 40:60 diet change. During the rest of the study, *IRS1* did not present significant changes in expression with dietary change for treatment B steers.

Urea Cycle

There was a significant treatment × diet change interaction for *SLC14A1* ($P < 0.01$) and a diet change effect ($P < 0.01$) (Table 1). Additionally, there was a trend for a diet interaction ($P = 0.10$). In treatment A steers, *SLC14A1* expression in general decreased with the addition of grain in the diet, with the exception of the 21 days under 100%

forage diet where *SLC14A1* had up-regulation. In treatment B steers, *SLC14A1* expression was inconsistent. There was a significant difference in *SLC14A1* expression at 21 days from the beginning of the study (Figure 13).

Discussion

Animal Performance

The body weight loss of the treatment A steers at the beginning of the study could be explained by an excessive refusal of the 100% forage diet that was administered. Also, steers in treatment A exhibited a "playing" behavior with the hay that worsened DMI issue. This behavior often resulted in the hay being out of the reach of the steer. After observing the important decrease in body weight illustrated in Figure 1, we started to administer the diet twice per day. Furthermore, after cleaning animals and pens, we decided to stay in the room ensuring the total consumption of the ration.

The rumen pH was within healthy limits throughout all the study in accordance with a similar study, despite a change in body weight and diet change (Lancaster *et al.,* 2015).

Histology

Health rumen papillae morphology is key to proper digestive physiology. More efficient cattle have been shown to have a thicker rumen epithelium compared to their less efficient counterparts (Lam *et al.,* 2017). The effects of a VFA can vary drastically depending on an animal's nutritional status and the rate at which VFAs are consumed

(Sakata. 1976, 1978). Throughout a ruminant animal's life, the types of feed ingested will affect rumen papillae differently. For instance, increasing the amount of concentrates in the diet, increases the rumen papillae length and density due to the increase of VFAs (Stobo *et al.,* 1966), specifically propionate and butyrate (Baldwin, 1998). Introduction of forage will cause papillae to be shorter (Castells *et al.,* 2013). Even early on, cattle fed high concentrate diets develop longer papillae which result in better beef quality (Reddy *et al.,* 2017). Measuring metabolizable energy can be a predictor of rumen papillae morphology. For example, young sheep fed a high ME diet had longer and wider papillae (Shen *et al.,* 2004). Our study showed similar results as those mentioned above. Treatment A steers had rumen papillae length has a relative increase from the beginning of the study to the end (i.e. with addition of grain over time). Specifically, there was a large increase with the switch from whole to cracked corn due to a potential greater availability of VFAs from the cracked corn. In contrast, treatment B had a slight decrease in rumen papillae length, after corn on the diet was switched from whole to cracked.

Rumen Epithelium Gene Expression

Cell Membrane Structure

CLDN1, associated with tight junctions, is a gene which had decreased expression with the decrease of grain proportion in the diet, which may be due to regeneration of papillae tissue. The forage to grain fed steers had a large increase in *CLDN1* expression with the

last couple of diet switches (40:60 and 20:80), which may be due to the increase in nutrients in need of shuttling. Furthermore, at the papillae level, *CLDN1* expression is stronger within the deeper layers, and a loss of surface cells would cause *CLDN1* to be enriched (Liu *et al.,* 2013). Previous studies have shown upregulation of *CLDN1* and *CLDN4* in low pH environments, both in cattle rumen epithelium (McCann *et al.,* 2016) and rat duodenum (Charoenphandhu *et al.,* 2008). In contrast, our results cannot be compared due to only moderate pH variations under healthy limits. The activation of *CDLN1* at the beginning of the study could represent an adaptive response to prevent a reduction in permeability during low feed intake (Pederzolli *et al.,* 2018).

Desmosomes are structures by which two adjacent cells are attached, formed from protein plaques in the cell membranes linked by filaments (Garrod, 1986). Desmosomes are composed of desmosome-intermediate filament complexes that can be broken into three regions: the extracellular core region, or desmoglea, the outer dense plaque, and the inner dense plaque (Delva *et al.,* 2009) The outer dense plaque contains the intracellular ends of desmocollin and desmoglein, this last one being the structure we were interested in analyzing. Desmoglein (*DSG1*) is a cell structure involved in cell-to-cell adhesion. Previous studies have shown disruption of tight junctions when cattle were switched to grain and developed SARA. It is hypothesized either increased permeability or paracellular transport may be one of the mechanisms by which SCFA are cleared from the rumen in response to the increased organic acid load imposed on the ruminal environment during SARA (Steele *et al*., 2011). *DSG1* has previously been shown to be

downregulated with high amounts of grain (Steele *et al.*, 2011). Our results seem to show down-regulation of *DSG1* due to decrease in body weight as compared to dietary changes. In conclusion, these results suggest that the structural integrity of the rumen epithelium might be compromised during periods of negative energy balance.

Fatty Acids

ACADSB, a gene associated with SCFA metabolism, has been shown to be more highly expressed in cows whose milk had a high milk fat content compared to those with lower milk fat (Jiang *et al.*, 2018). The forage to grain steers' *ACADSB* gene expression increased sharply at the end of the study; possibly the delay in increased expression may be related to a threshold of FAs not met until the highest grain diet was provided. In fact, we speculate that our results for *ACADSB* may indicate that ruminal epithelium is likely more reliant on oxidation of short-and branched-chain FA to derive energy because this gene accounted for almost 9% of total mRNA measured (Additional Table 1). Our results complement those of Naeem *et al.* 2012 who found increases of *ACADSB* expression with higher plains of nutrition in milk replacer fed dairy calves. *CPT1A*, which catalyzes the entry of LCFA into mitochondria (Zammit, 1984), has been shown to be stably expressed in the rumen epithelium despite varying milk replacer diets (Naeem *et al.*, 2012). Our results showed similar trends in both treatments which agrees with Naeem *et al.* 2012. Together, the observed *ACADSB* and *CPT1A* expression patterns in the present study, may imply the rumen is less dependent on LCFAs than SCFAs and/or BCFAs.

However, one previous study showed *CPT1A* expression increases in ruminants with increases of NEFAs, whose levels are different between grains and forages (Xu *et al.*, 2011).

Glycolysis

PFKL is associated with glycolysis and has been shown to be affected by changes in dietary fat, but not carbohydrates (Seip *et al.*, 2008). At the end of the period when treatment B steers received the 60:40 diet for 16 days, glycolysis appears to be a crucial compensatory mechanism for a period of increased energy demand, which could be filled by ATP-generating processes like glycolysis (Laarman, 2015). Although, we cannot determine the reason for this *PFKL*'s peak at the specified situation. Finally, *PFKL* has also been shown to have higher expression in low RFI cattle, indicating greater energy production. This may make *PFKL* a good gene to select for feed efficiency (Kong *et al.*, 2016).

Ketogenesis

ACAT1 catalyzes the first step in ketogenesis (Lane *et al.*, 2012). In our study, during the inefficient feed consumption experienced in treatment A steers at the beginning of the study, signs of ketogenesis were noticed by a significant increment in *ACAT1* expression. Previously it has been shown that bulls fed a high concentrate diet increased absorption of acetic acid due to greater *ACAT1* activity in rumen epithelium (Harmon *et al.* 1991); our results for treatment A concur with this. It has been noted that *ACAT1* expression

decreases late in fattening (Lee *et al.,* 2007), which is contradictory to what we observed with a gain in body weight and increase in *ACAT1* expression the last 16 days of the study.

HMGCL contributes to ketogenesis by converting HMG-CoA to acetoacetate (Lane *et al.,* 2002) and *HMGCS2* encodes the rate-limiting enzyme in the synthesis of ketone bodies (Ma *et al.,* 2017). *HMGCS2* plays a central role in coordinating ruminal ketogenic flux (Naeem *et al.*, 2012). During periods of positive energy balance, beta-hydroxybutyric acid is produced primarily via ketogenesis from butyrate within the rumen epithelial cells. As the rate-limiting enzyme in the synthesis of ketone bodies, the *HMGCS2* protein plays an important role in the metabolism of VFA within the rumen epithelium (Ma *et al.*, 2017). In the rumen papillae of Angus-Hereford crossbred heifers, *HMGCL* expression increase with larger amounts of grain in animals' whose pH increased with the introduction of more grain on the diet (Zhao *et al.,* 2017). In primiparous dairy cows fed diets with different NDF to starch ratios, the expression of *HMGCS2* was downregulated with increasing NDF to starch ratio (Ma *et al.,* 2017). In the rumen of sheep, both *HMGCL* and *HMGCS2* were shown to be upregulated in lambs fed a starter diet in comparison to those that nursed (Wang *et al.,* 2016). In our study, like Wang *et al.* 2016, treatment A steers had increased expression of both *HMGCL* and *HMGCS2* as the proportion of grain fed increased, denoting a greater degree of ketogenesis.

Lactate/pyruvate metabolism

Pyruvate is an important intermediate in key pathways of energy metabolism. *LDHA* is a cytoplasmic enzyme involved in the reversible catalysis of anaerobic glycolysis to produce lactate from pyruvate. Increased expression of *LDHA* during nutrient restriction suggests an increase in lactate production. Lactate production occurs when oxygen levels are low and, it can be necessary in order to regenerate NAD+, which is consumed in the synthesis of pyruvate from glucose, ensuring that energy production is maintained (O'Shea *et al.,* 2016). In accordance with this, our data showed that treatment A steers had an increase in *LDHA* expression when the animals were losing body weight during the first 21 days under study. Furthermore, in another study, ruminal *LDHA* levels decreased in calves fed an enhanced plane of nutrition, possibly leading to greater utilization of circulating amino acids for gluconeogenesis by ruminal tissue (Naeem *et al.,* 2014).

Oxidative Stress

Oxidative stress is an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants (Celi, 2011). *NQO1* protects cells from damage caused by oxidative stress by acting as a substrate for the two-electron transferring flavoenzymes (Sarlauskas *et al.,* 2004). In the rumen papillae of Angus × Hereford steers, *NQO1* had increased expression in low RFI individuals, suggesting a greater clearance of oxidative damage (Kern *et al.,*

2017). Additionally, in cattle fed grain to the point of subacute ruminal acidosis (SARA), *NQO1* expression was downregulated (Abaker *et al.,* 2017). Our results showed that *NQO1* expression increased with the addition of grain. In our study, *NQO1* had significant activation when there was a significant change on the diet (i.e., when whole corn was replaced with cracked corn and when 80% of the diet was grain-base) (Figure 10). Nevertheless, our steers maintained a healthy rumen pH throughout the duration of the study.

Tissue Growth/Development

The intake of protein- and energy-rich diets promotes the growth of ruminal tissues by promoting epithelial cell proliferation (Shen et al., 2004). *AKT3*, via cell proliferation, can coordinate an increase in organ weight in mice (Dummler *et al.,* 2006). However, it has been found that calves with enhanced early plane of nutrition initially express lower amounts of *AKT3* before it increased at the end of the mild-fed period (Naeem *et al.,* 2012). This expression pattern (i.e., an initial increase followed by a decrease) is consistent with our results for treatment A steers.

EGFR is associated with tissue growth and structure. It has been found to be a focal regulatory gene in the adaptive response of rumen papillae in dairy cattle during early lactation (Steele et al., 2015). However, in Holstein calves fed either a calf starter diet with or without hay, rumen epithelium expression of *EGFR* was not significantly

different (Kim *et al.*, 2016). The significant increase in expression of *EGFR* in treatment A steers may be attributable to the rumen remodeling with the changes in diet.

Epiregulin (*EREG*) is also associated with tissue growth and structure. *EREG* acts as a ligand for both epidermal growth factor receptor (*EGFR*) and the erb-b2 receptor tyrosine kinase 4 (*ERBB4*). *EREG* is believed to be involved in a variety of processes including inflammation, wound healing, and cell proliferation (Toyoda et al., 1997). In mice, it has been shown to modulate intestinal stem cell proliferation (Gregorieff *et al.*, 2015). In rumen papillae, both *EREG* and *EGFR* expression increased as Holstein cows progressed from day 10 to day 28 in milk, indicating ruminal papillae proliferation (Minuti et al., 2015). In our study, *EREG* expression did not present a clear pattern of response to the change in diets.

The diet composition affects the growth of rumen epithelial cells. Genes that belong to the IGF family play a role in the development of rumen epithelial cells (Shen *et al.*, 2004). An increase in the concentration of butyrate or high level of digestible carbohydrates in the diet increased *IGFBP*'s activation and led to greater proliferation and differentiation of rumen epithelial cells (Ma *et al.*, 2017). *IGFBP5* is also associated with cellular apoptosis, proliferation, and differentiation specifically in mammary tissue (Mohapatra *et al.*, 2015). Expression of *IGFBP5* mRNA has been shown to be unregulated in the rumen papillae of sheep supplemented a higher plane of nutrition and in cattle transitioning to a high grain diet (Jing *et al.*, 2018; Steele *et al.*, 2011; Steele *et*

al., 2012). Like in these studies, our results show an increase in *IGFBP5* expression as grain proportion was increased in the diet (Treatment A) and a not clear pattern when diet decrease in grain proportion (Treatment B). *IGFBP5* results suggest that the rumen epithelium cells might present higher degree of proliferation with increments in grain content in the diet.

The IRS proteins are a family of cytoplasmic adaptor proteins that transmit signals from the insulin and IGF-1 receptors to elicit a cellular response (Shaw *et al.*, 2011). It is important to mention that *IRS1* mRNA expression has been previously shown as an indicator of *IRS-1* signaling, but its effectiveness is disputed (Araki *et al.*, 1994; Sun *et al.*, 1991; Sun *et al.*, 1992). *IRS-1* and *IRS-2* are ubiquitously expressed and are the primary mediators of insulin-dependent mitogenesis and regulation of glucose metabolism in most cell types (White, 2002). In the rumen epithelium of multiparous Holstein cows, *IRS1* has been shown to increase with a transition to a higher energy, lower straw diet. The authors surmise that this effect may increase insulin sensitivity (Minuti *et al.*, 2015). The reaction of *IRS1* expression to the 20:80 diet on steers from treatment B coincided with previous results but we cannot explain the lack of *IRS1* expression in steers from treatment A.

Urea Cycle

Solute carrier family 14 member 1 (*SLC14A1*) mediates the basolateral cell membrane transport of urea across the rumen epithelium (Abdoun *et al.*, 2010; Stewart *et al.*, 2005).

In the rumen, urea is turned into ammonia, which is used by rumen microbes as a nitrogen source. As these microbes turn over and are digested in the intestine, this nitrogen returns to the animal as microbial cell protein, an important energy source (Abdoun, Stumpff, & Martens, 2006). *SLC14A1* has been shown to be upregulated by solid feed intake; this may be because of urea recycling (Berends *et al.,* 2014). In the rumen epithelium of sheep, *SLC14A1* was not differentially expressed as compared to different compartments of the gastrointestinal tract (Xiang *et al.,* 2016). Although, it has been demonstrated that *SLC14A1* expression is upregulated in the rumen epithelium of sheep fed a high concentrate diet (Lu *et al.,* 2014). In our results, mRNA expression of *SCL14A1* seems to be increased under conditions of diet restriction (i.e., body weight loss) or change on grain proportion in the diet, consistent with expected changes in urea transport across the rumen epithelial wall during negative energy balance.

Summary and Conclusions

Rumen epithelium gene expression results indicated that the majority of the genes tested responded to transitioning diets. Significant differences in gene expression occurred with time due to the variation in the proportion of grain in the diet. A decrease in body weight in treatment A steers at the beginning of the study due to an excessive feed refusal was reflected in altered expression of genes related to rumen epithelium cell integrity (*DSG1* and *CLDN1*), fatty acids metabolism (*CPT1A* and *ACADSB*), ketogenesis (*HMGCL, HMGCS2* and *ACAT1*) and urea cycle (*SCL14A1*). Furthermore,

using cracked corn as opposed to whole corn-based diets also had a significant effect on the expression of genes related to fatty acid metabolism (*ACADSB*), ketogenesis (*HMGCS2* and *ACAT1*) and oxidative stress (*NQO1*) in treatment A steers. In Treatment A steers, a relative increase in rumen papillae length was also observed. Studies looking further into these genes as well as others deserve more attention.

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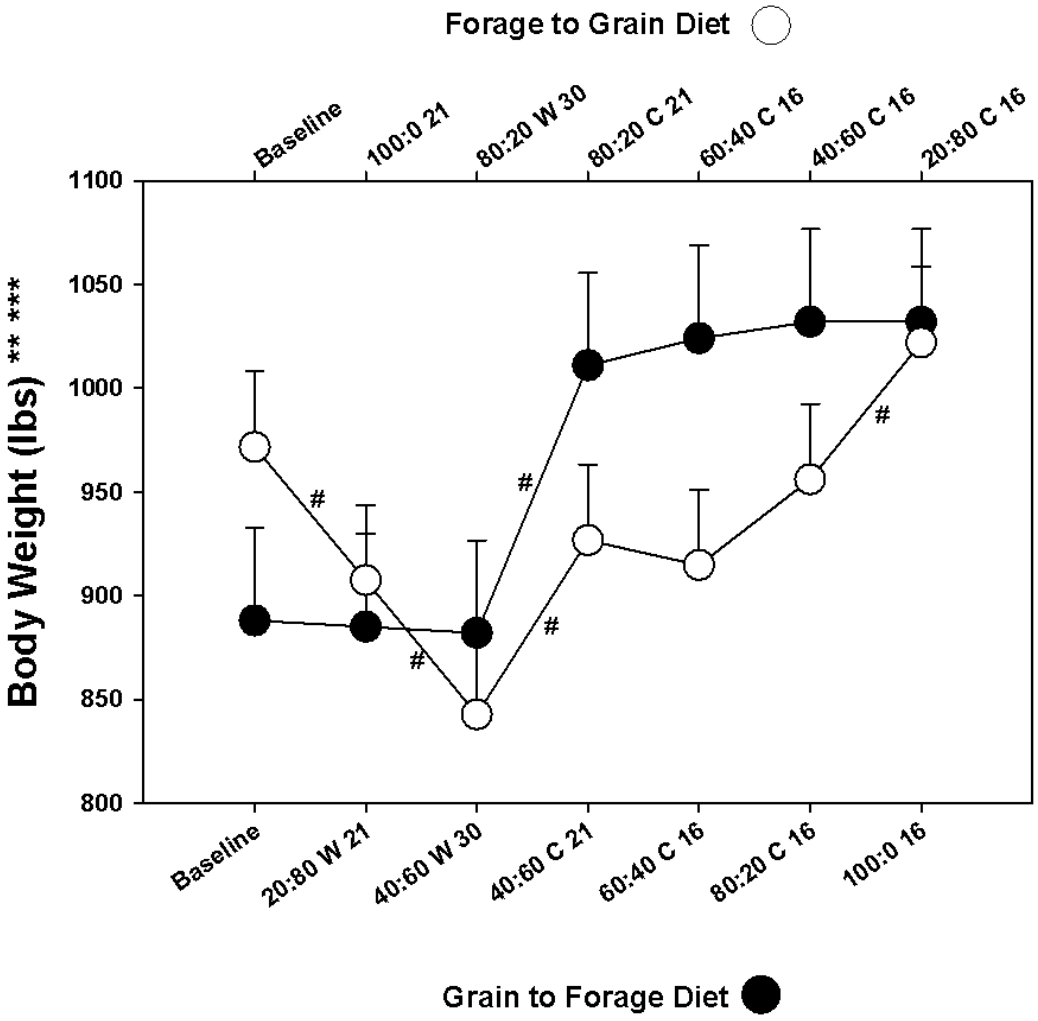
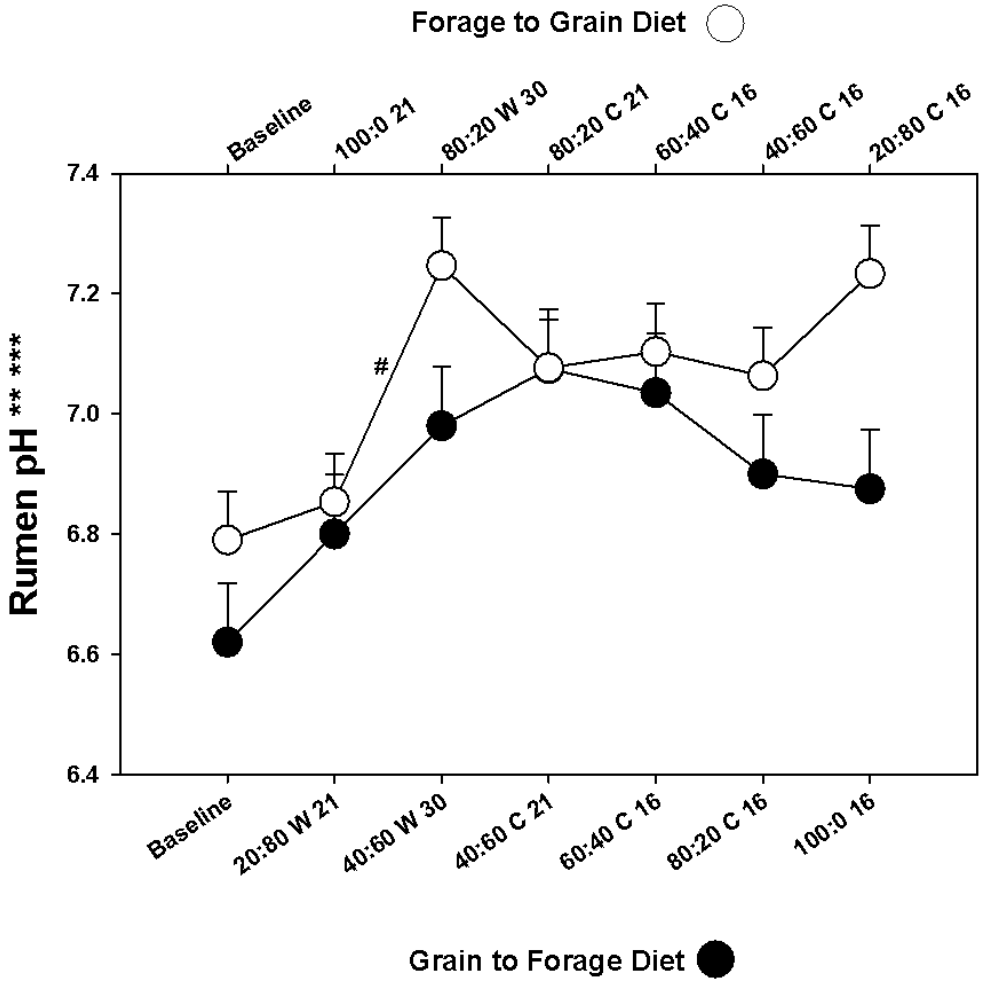
Figure 1. Jersey steers body weights from treatment A (from forage to grain, n = 3) and steers from treatment B (from grain to forage, n = 2) through the study.



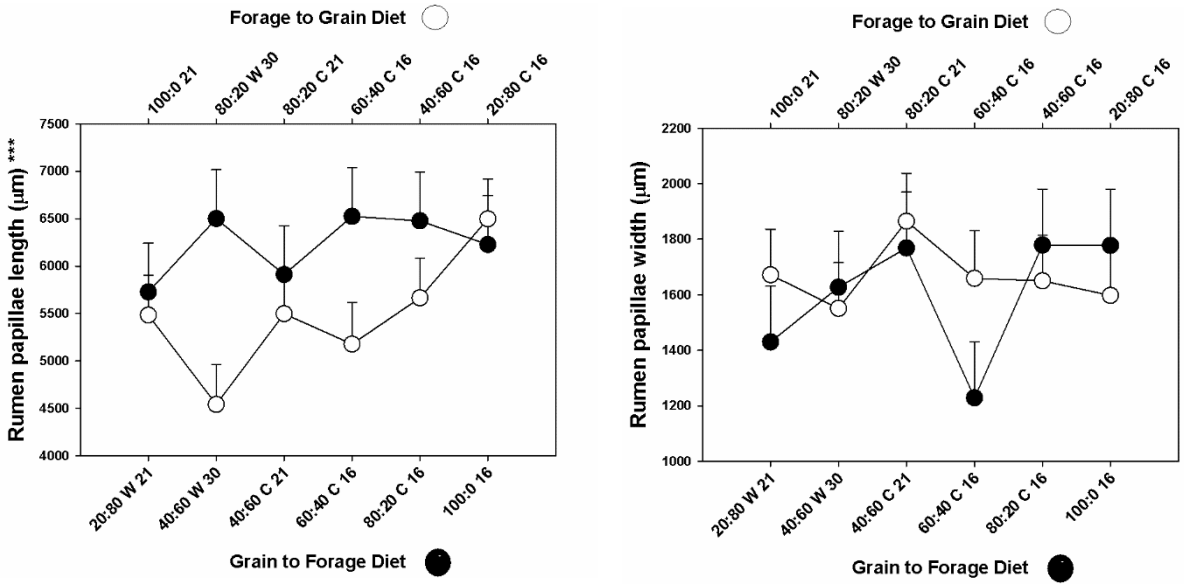
Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × diet change interaction (***), diet change effect (**) and treatment effect (*).Symbols (#) on lines denote significant differences ($P < 0.05$) between two time point for the same treatment.

Figure 2. Jersey steers rumen liquid pH from treatment A (from forage to grain, n = 3) and steers from treatment B (from grain to forage, n = 2) through the study



Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × diet change interaction (***), diet change effect (**) and treatment effect (*). Symbols (#) on lines denote significant differences ($P < 0.05$) between two time point for the same treatment.

Figure 3. Rumen papillae length and width in Jersey steers from treatment A (from forage to grain) and steers from treatment B (from grain to forage)



Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × diet change interaction (***), diet change effect (**) and treatment effect (*).

Figure 4. Relative mRNA abundance of genes analyzed in Jersey steers rumen epithelium papillae.

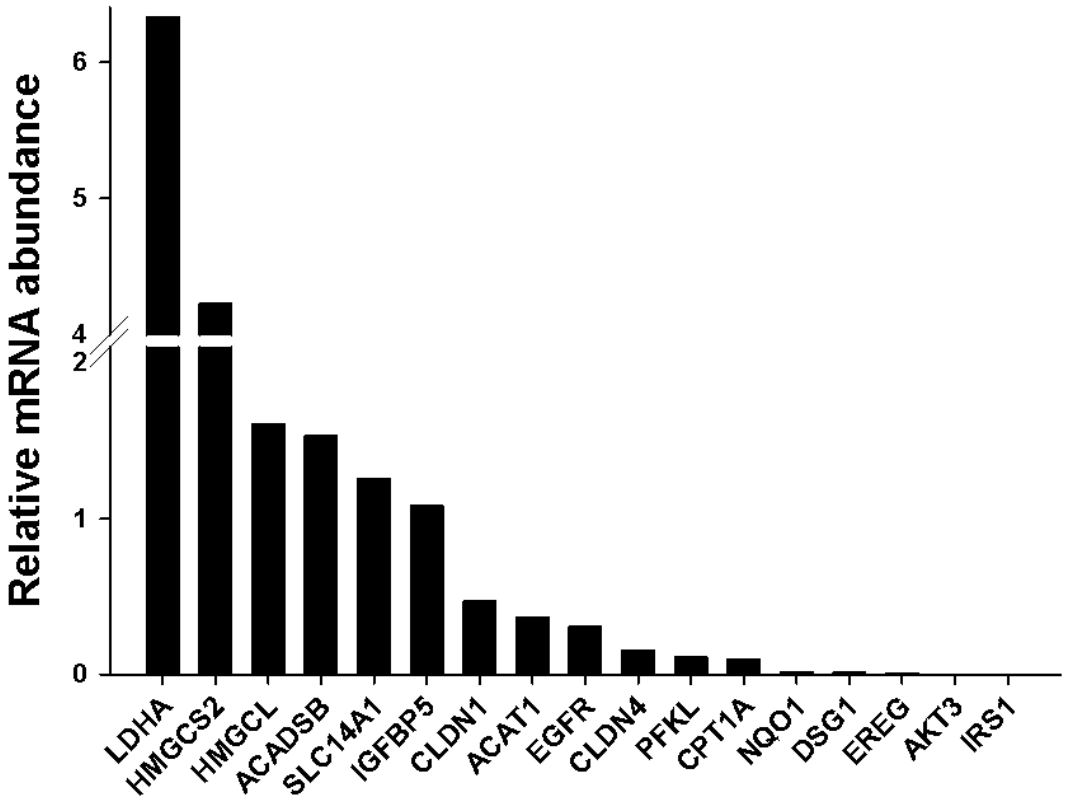
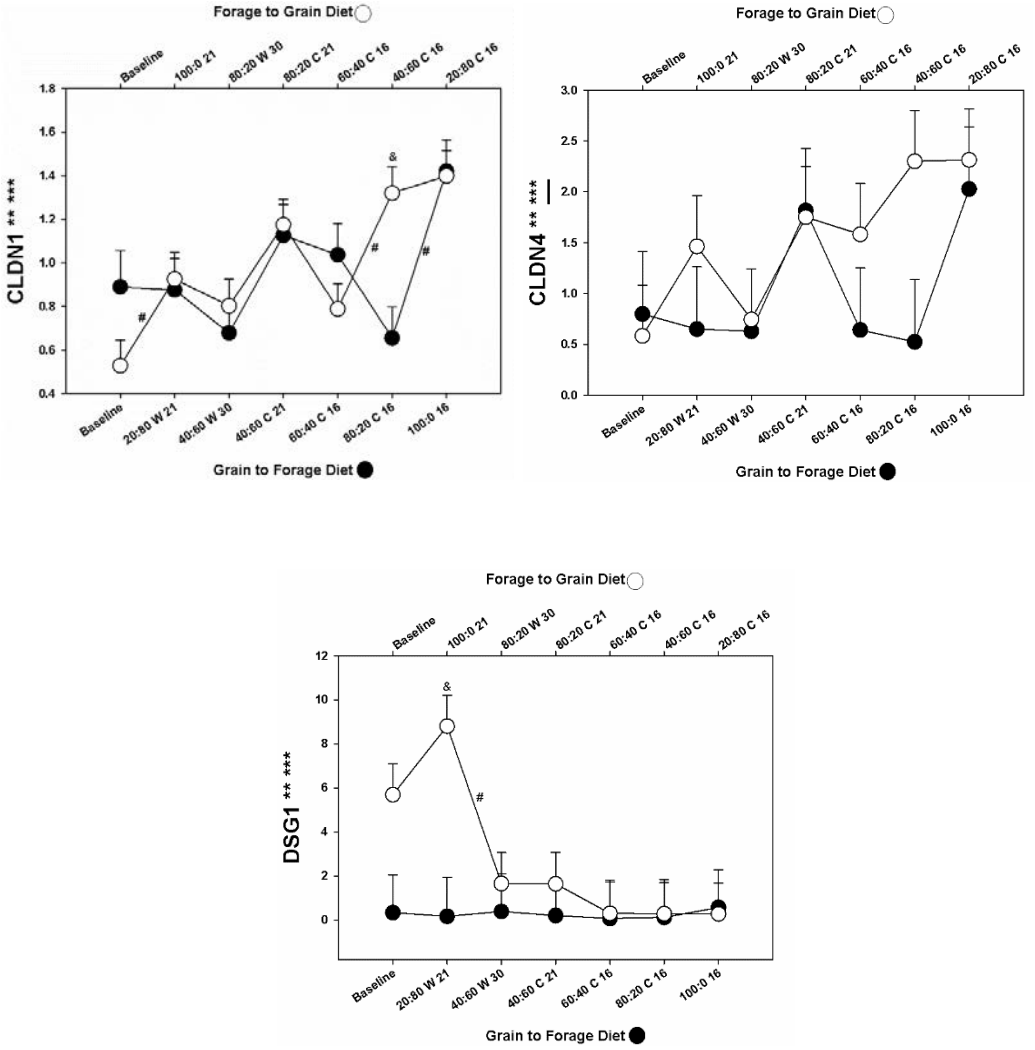


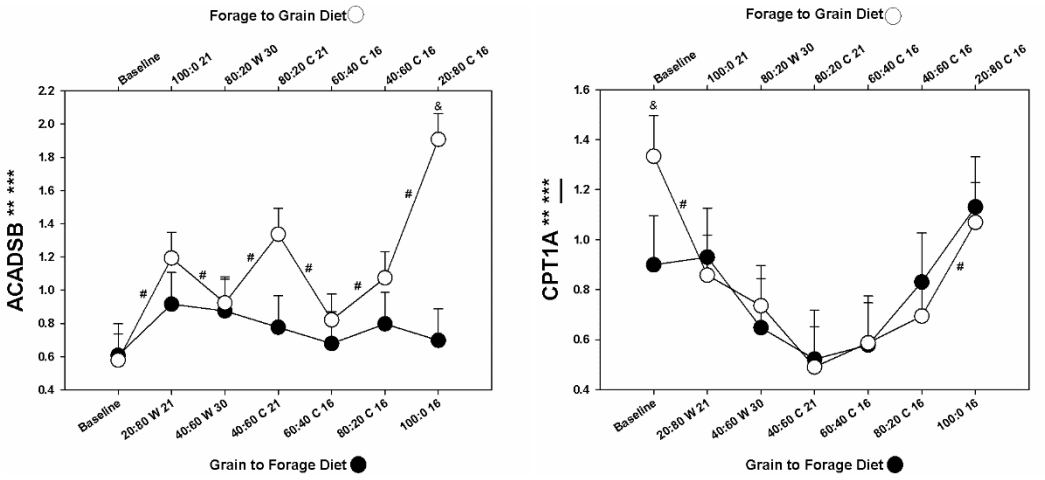
Figure 5. Expression of genes related to cell membrane structure in rumen epithelium papillae in Jersey steers from treatment A (from forage to grain) and steers from treatment B (from grain to forage).



The first biopsy was taken when treatment A started to receive a 100% forage diet (100:0) and treatment B finished the adaptation period to a 20:80 whole corn-based diet (baseline). Second biopsy was taken after steers from treatment A where on 100:0 for 21 days (100:0 21) and they switched to 80:20 and, when steers from treatment B where on a 20:80 whole corn-based diet for 21 days (20:80 W 21) and they switched to a 40:60 diet. Third biopsy was taken after steers from treatment A where on a 80:20 whole corn-based diet for 30 days (80:20 W 30) and they switched to a 80:20 cracked corn-based diet and, when steers from treatment B where on a 40:60 whole grain-based diet for 30 days (40:60 W 30) and they switched to a 40:60 cracked corn-based diet. Fourth biopsy was taken when steers from treatment A where on a 80:20 whole grain-based diet for 30 days (80:20 W 30) and they switched to a 80:20 cracked grain-based diet and. steers from treatment B where on a 40:60 cracked corn-based diet for 21 days (40:60 C 21) and they switched to a 60:40 cracked corn based diet. Fifth biopsy was taken when steers from treatment A where on a 60:40 cracked

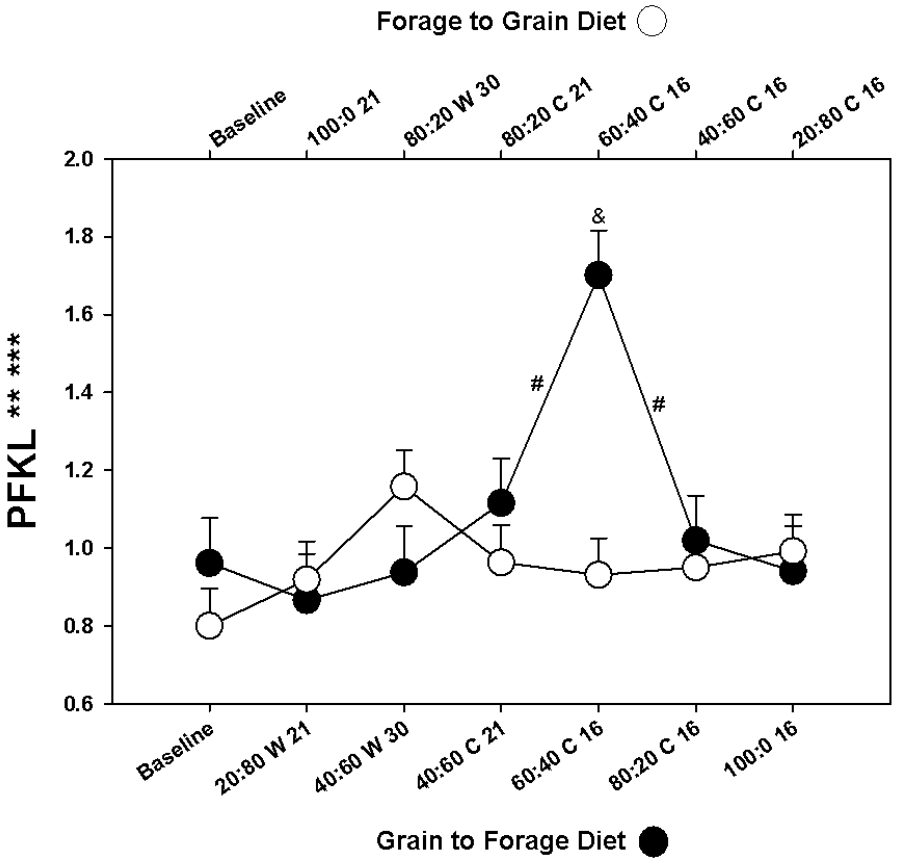
corn-based diet for 16 days (60:40 C 16) and they switched to a 40:60 cracked corn-based diet and, when steers from treatment B where on a 60:40 cracked corn-based diet for 16 days (60:40 C 16) and they switched to a 80:20 cracked corn-based diet. Sixth biopsy was taken when steers from treatment A where on a 40:60 cracked corn-based diet for 16 days (40:60 C 16) and they switched to a 20:80 cracked corn-based diet and steers from treatment B where on a 80:20 cracked corn-based diet for 16 days (80:20 C 16) and they switched to a 100:0 diet. Seventh biopsy was taken when steers from treatment A where on a 20:80 cracked corn-based diet for 16 days (20:80 C 16) and steers from treatment B where on a 100:0 diet for 16 days (100:0 16). Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × diet change interaction (***), diet change effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (#) on lines denote significant differences ($P < 0.05$) between two time point for the same treatment, symbols (&) denote significant differences ($P < 0.05$) between treatment at the same time point.

Figure 6. Expression of genes related to fatty acid metabolism in rumen epithelium papillae in Jersey steers from treatment A (from forage to grain) and steers from treatment B (from grain to forage).



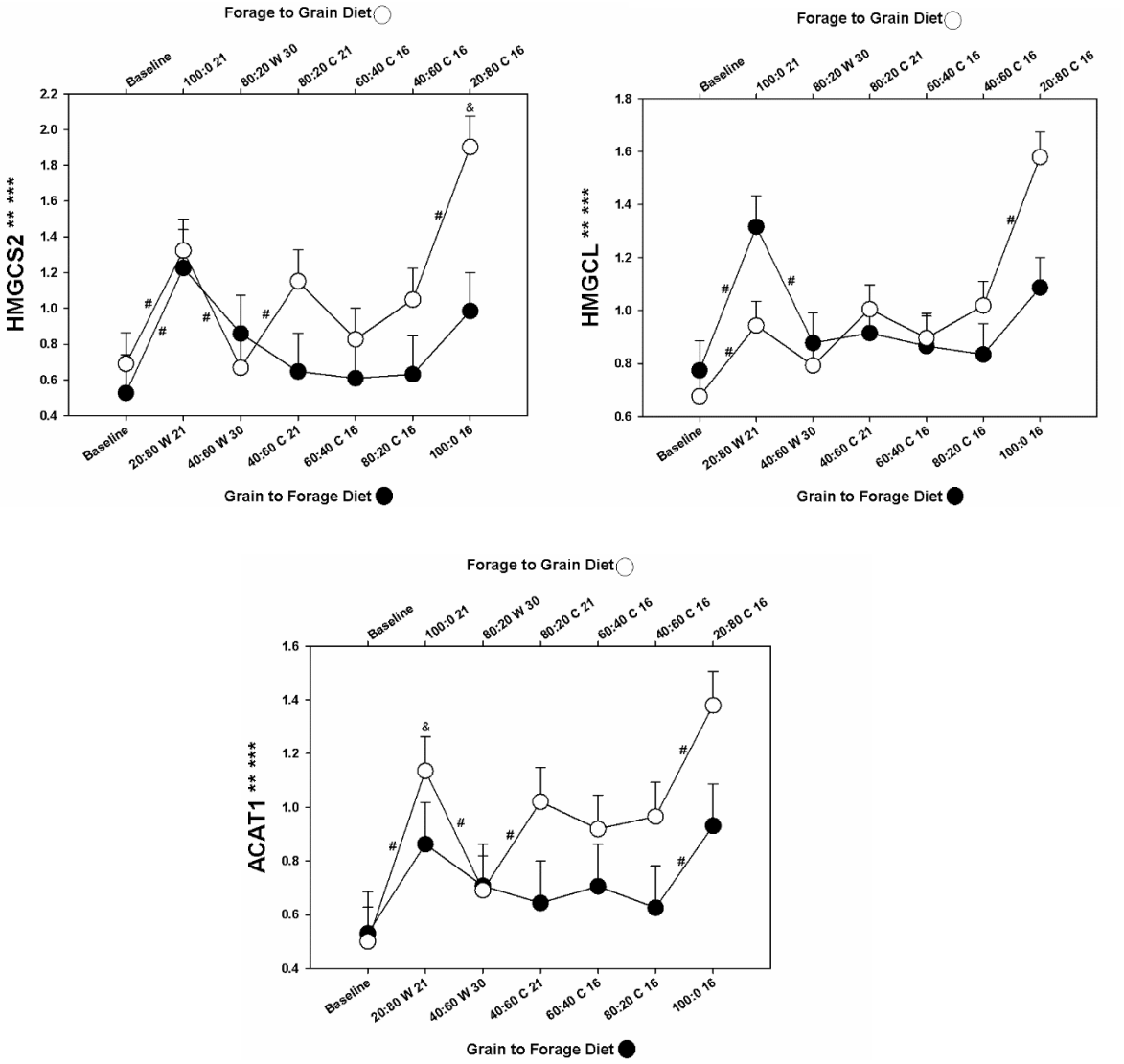
Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × diet change interaction (***), diet change effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (#) on lines denote significant differences ($P < 0.05$) between two time points for the same treatment, symbols (&) denote significant differences ($P < 0.05$) between treatments at the same time point.

Figure 7. Expression of genes related to glycolysis in rumen epithelium papillae in Jersey steers from treatment A (from forage to grain) and steers from treatment B (from grain to forage).



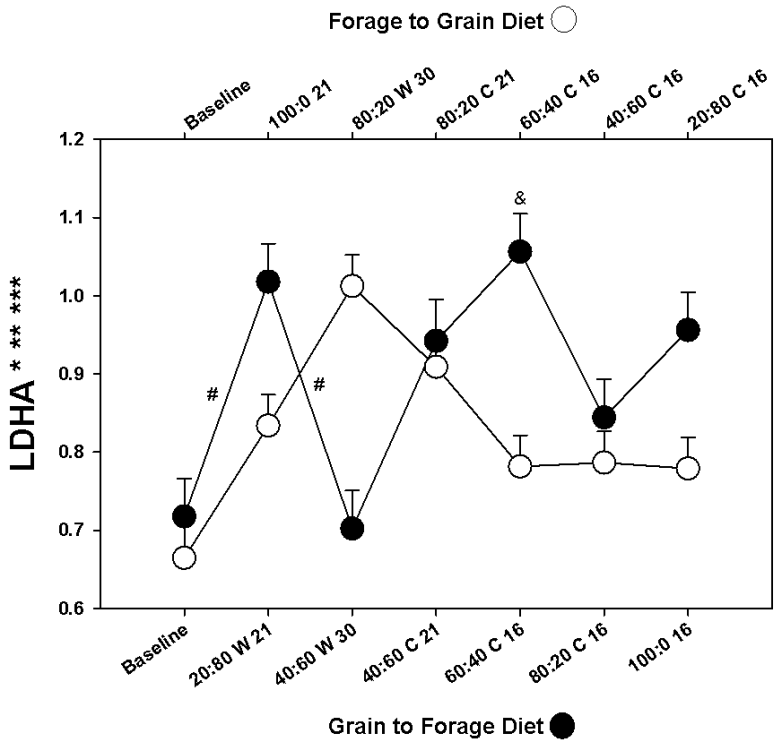
Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × diet change interaction (***), diet change effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (#) on lines denote significant differences ($P < 0.05$) between two time points for the same treatment, symbols (&) denote significant differences ($P < 0.05$) between treatments at the same time point.

Figure 8. Expression of genes related to ketogenesis in rumen epithelium papillae in Jersey steers from treatment A (from forage to grain) and steers from treatment B (from grain to forage).



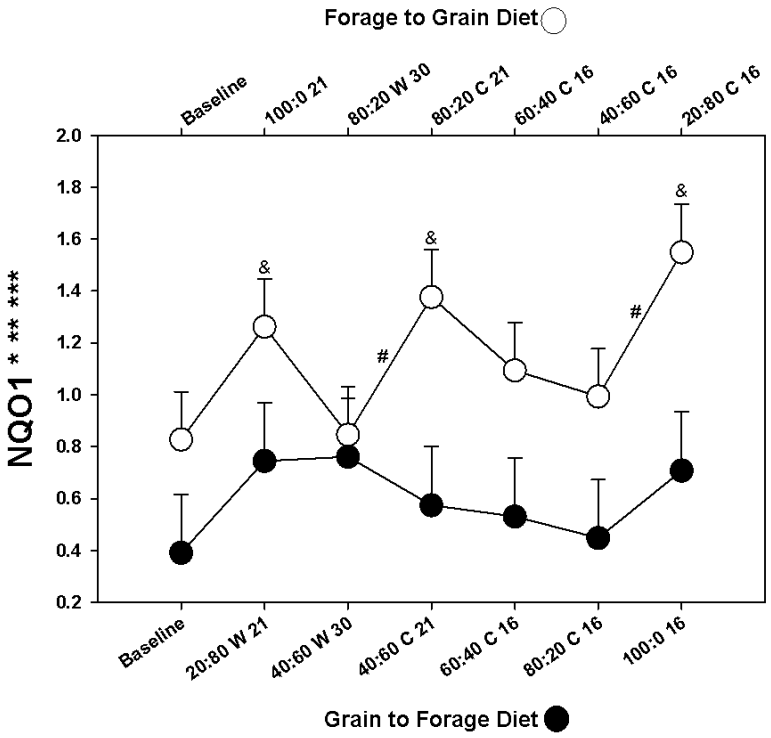
Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × diet change interaction (***), diet change effect (**) and treatment effect (*). Symbols (#) on lines denote significant differences ($P < 0.05$) between two time points for the same treatment, symbols (&) denote significant differences ($P < 0.05$) between treatments at the same time point.

Figure 9. Expression of genes related to lactate/pyruvate in rumen epithelium papillae in Jersey steers from treatment A (from forage to grain) and steers from treatment B (from grain to forage).



Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × diet change interaction (***), diet change effect (**) and treatment effect (*). Symbols (#) on lines denote significant differences ($P < 0.05$) between two time points for the same treatment, symbols (&) denote significant differences ($P < 0.05$) between treatments at the same time point

Figure 10. Expression of genes related to oxidative stress in rumen epithelium papillae in Jersey steers from treatment A (from forage to grain) and steers from treatment B (from grain to forage).



Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × diet change interaction (***), diet change effect (**) and treatment effect (*). Symbols (#) on lines denote significant differences ($P < 0.05$) between two time points for the same treatment, symbols (&) denote significant differences ($P < 0.05$) between treatments at the same time point.

Figure 11. Expression of genes related to tissue growth and development in rumen epithelium papillae in Jersey steers from treatment A (from forage to grain) and steers from treatment B (from grain to forage).

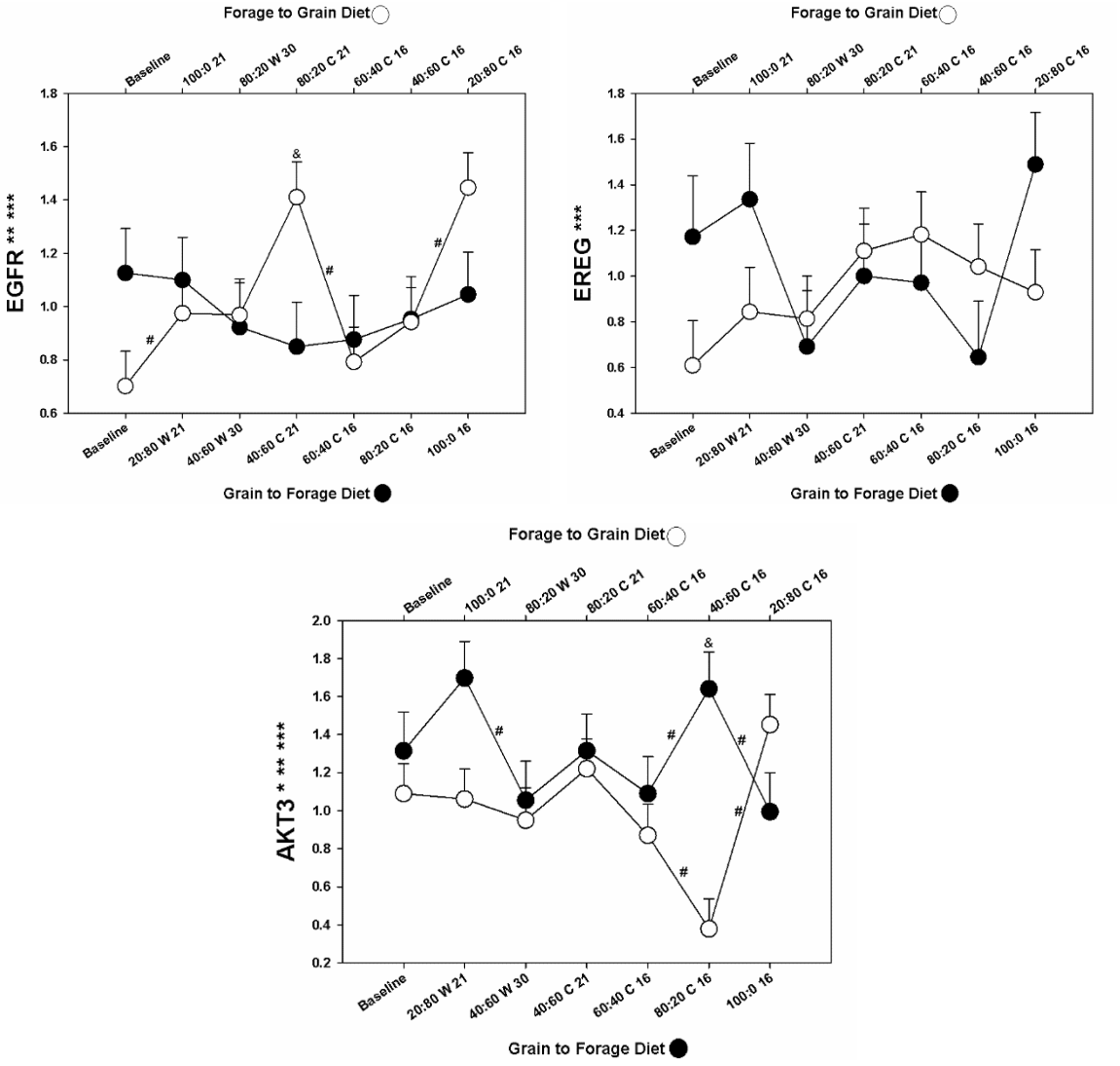
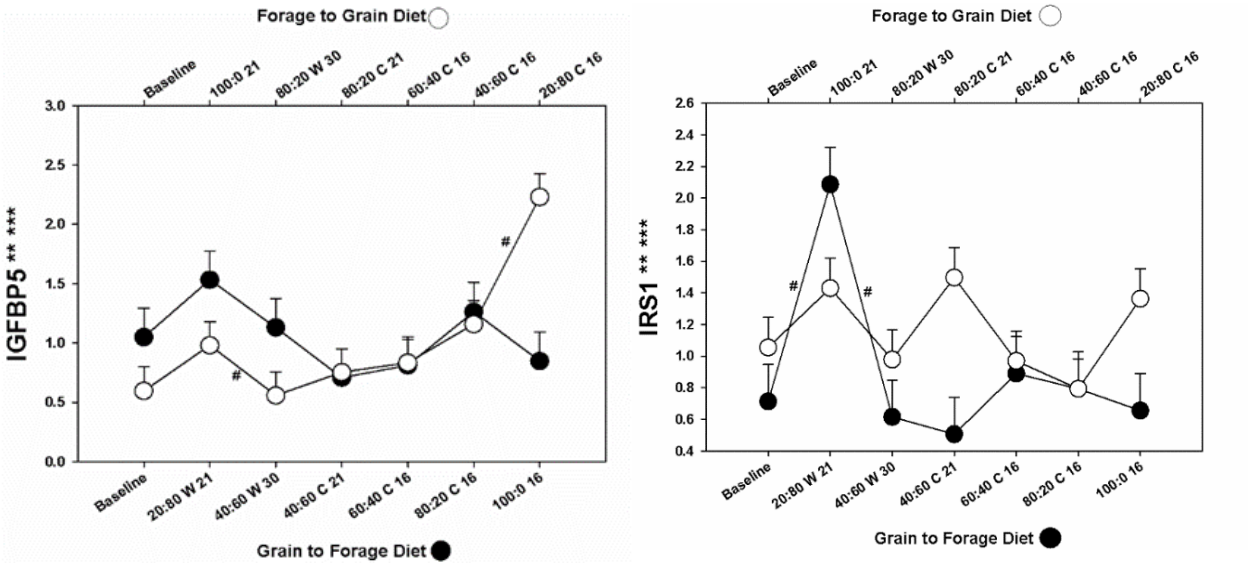
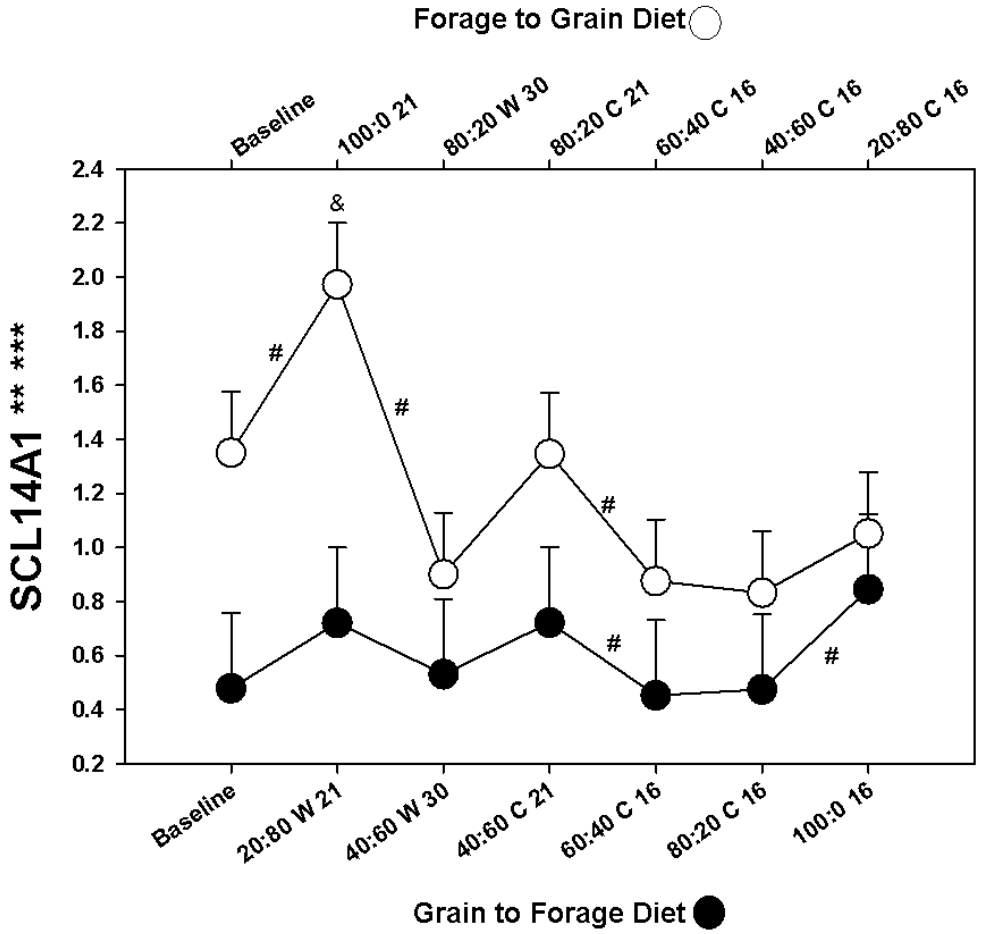


Figure 11. Continuation



Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × diet change interaction (***), diet change effect (**) and treatment effect (*). Symbols (#) on lines denote significant differences ($P < 0.05$) between two time points for the same treatment, symbols (&) denote significant differences ($P < 0.05$) between treatments at the same time point.

Figure 12. Expression of genes related to urea cycle in rumen epithelium papillae in Jersey steers from treatment A (from forage to grain) and steers from treatment B (from grain to forage).



Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × diet change interaction (***), diet change effect (**) and treatment effect (*). Symbols (#) on lines denote significant differences ($P < 0.05$) between two time points for the same treatment, symbols (&) denote significant differences ($P < 0.05$) between treatments at the same time point.

Table 1. Overall least mean squares values for expression of genes analyzed in rumen epithelium papillae of Jersey steers from treatment A (from forage to grain, F:G) and treatment B (from grain to forage, G:F).

Genes	Treatments		SEM	P values		
	F:G	G:F		Treatment	Diet change	Trt. × diet change
ACADSB	1.12	0.76	0.18	0.20	0.0001	0.0001
ACAT1	0.94	0.72	0.14	0.23	0.0001	0.0009
AKT3	1.00	1.30	0.14	0.04	0.0002	0.0001
CLDN1	0.99	0.95	0.09	0.79	0.0001	0.0002
CLDN4	1.53	1.01	0.33	0.58	0.0074	0.0571
CPT1A	0.82	0.79	0.18	0.92	0.0001	0.0672
DSG1	2.66	0.26	1.49	0.12	0.0001	0.0001
EGFR	1.03	0.98	0.13	1.00	0.0001	0.0001
EREG	0.93	1.04	0.14	0.37	0.3726	0.001
HMGCL	0.99	0.95	0.10	0.81	0.0001	0.0001
HMGCS2	1.09	0.78	0.19	0.24	0.0001	0.0003
IGFBP5	1.02	1.05	0.21	0.64	0.0001	0.0001
IRS1	1.16	0.89	0.11	0.15	0.0005	0.0019
LDHA	0.82	0.89	0.02	0.02	0.0001	0.0001
NQO1	1.14	0.59	0.19	0.01	0.0001	0.0416
PFKL	0.96	1.08	0.10	0.40	0.0001	0.0001
SLC14A1	1.19	0.60	0.27	0.10	0.0001	0.0002

Statistical significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1.

Additional Table 1. RT-qPCR performance table among the 17 genes measured in rumen papillae on Jersey steers.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency (%)[5]	relative mRNA abundance[6]	1/EΔCt[7]	%
ACADSB	19.500	-0.468	-2.529	0.998	148.6	1.532	0.087	8.71
ACAT1	21.103	1.176	-2.704	0.999	134.3	0.367	0.021	2.09
AKT3	25.711	5.675	-2.389	0.996	162.2	0.004	0.000	0.02
CLDN1	20.856	0.836	-2.554	0.996	146.3	0.471	0.027	2.68
CLDN4	22.085	2.080	-2.547	0.999	147.0	0.153	0.009	0.87
CPT1A	22.765	2.575	-2.521	0.991	149.3	0.095	0.005	0.54
DSG1	23.883	3.931	-2.174	0.996	188.4	0.016	0.001	0.09
EGFR	21.380	1.327	-2.567	0.998	145.2	0.304	0.017	1.73
EREG	25.651	5.634	-2.549	0.993	146.8	0.006	0.000	0.04
HMGCL	19.442	-0.525	-2.540	0.998	147.6	1.609	0.092	9.15
HMGCS2	18.293	-1.701	-2.717	0.999	133.4	4.228	0.240	24.04
IGFBP5	19.863	-0.086	-2.583	0.998	143.9	1.079	0.061	6.14
IRS1	26.445	6.464	-2.420	0.999	159.0	0.002	0.000	0.01
LDHA	17.818	-2.185	-2.727	0.998	132.7	6.330	0.360	36.00
NQO1	24.605	4.637	-2.598	0.999	142.6	0.016	0.001	0.09
PFKL	22.393	2.423	-2.570	0.998	145.0	0.114	0.006	0.649
SLC14A1	19.832	-0.258	-2.594	0.999	142.9	1.257	0.071	7.14
Total						17.584	1.000	100.000

[1] The median is calculated considering all time points and all steers, [2] The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each steer, [3] Slope of the standard curve, [4] R^2 stands for the coefficient of determination of the standard curve, [5] [10^(-1/slope)]-1 x 100, [6] relative mRNA abundance = 1/ Efficiency Median ΔCt, [7] 1/EΔCt = relative mRNA abundance/∑relative mRNA abundance

Additional Table 2. GenBank accession number, gene ID, sequence, starting point and amplicon size (bp) of primers for *Bos taurus* used to analyze gene expression in rumen epithelium by RT-qPCR.

Accession #	Gene	Forward Primer	F-start	Reverse Primer	R-Start	BP
NM_001017933.1	ACADSB	GCGCTGCTACGAAGAAACTTC	193	TTGTCGCTTTGAGCTGAGCTT	292	100
NM_001046075.1	ACAT1	GCGGAGCGAAGTTATGTATCAAA	93	GAGAGGAAAGACTGCCTAGAAAGGA	192	100
XM_019976103.1	AKT3	GGGAGAGCTGTTTTTCCATTTG	731	ATGTAGATAGTCCAAGGCAGAGACAA	830	100
AB178476.1	CLDN1	CCGTTGGCATGAAGTGTATGA	517	GCCAGACCTGAAATAAGAAAGATCA	616	100
NM_001014391.2	CLDN4	CCCAGCCAGCAACTACGTCTA	696	TCACAGATTGCAGTGAGCTCAGT	797	102
NM_001304989.1	CPT1A	AAATCCTGGTGGGCTACCAAT	809	TCGCGAAGTAGTTGCTGTTCA	908	100
NM_174045.1	DSG1	AAGGTTGTTAAGCCCCTAGATTTTG	1094	ATTGCAGTTGCTGTGAGTTTATATTGA	1215	122
XM_592211.8	EGFR	TCAGACCATTGCCTTTAATTGAGA	4609	TGACCTCCAAAGACTTGATATACTTCCT	4709	101
XM_019962926.1	EREG	CAAAGTCAAAGCAAGGAGAGAGATG	2586	GACACCTCAGGGCAGTTGAATT	2685	100
NM_001034034.2	GAPDH	CACTCACTCTTCTACCTTCGATGCT	932	CCCTGTTGCTGTAGCCAAATTC	1035	104
NM_001075132.1	HMGCL	GTACGTCTCCTGTGTGCTTGGA	519	CTCGTAGCAGCCCATGGAGTA	618	100
NM_001045883.1	HMGCS2	TCTGGTTTGGCAGCAAGTTTCT	1290	TTCTGCAGGTCTGATGTACTGGAT	1390	101
XM_010802317.1	IGFBP5	ATACTAGATGGTAGGTGTTCCTAAAGCA	1776	ACTATCAGAGTGTGGATTGATTTCTCA	1880	105
XM_003581871.1	IRS1	TGTTGACTGAACTGCACGTTCT	4268	CATGTGGCCAGCTAAGTCCTT	4379	112
BC146210.1	LDHA	TGTTGTTTCCAATCCAGTCGATAT	560	GGAAGCGAGCTGAATCCAGAT	669	110
NM_001034535.1	NQO1	CAATCCCGTCATCTCCAGAAA	242	TCAGACGGCCTTCTTTATAAGCTAA	345	104
NM_001080244.2	PFKL	CGAGAAGATGAAGACAGAGATCCA	1860	GGAGGAGTAGAGGTTGTACAGGAACT	1959	100
NM_001008666.1	SLC14A1	TATGTCCATGACGTGTCCAGTCT	515	GTACATTGACAACGCCATATTGAAG	620	106
NM_001037471.2	UXT	CTGGCAGAAGCTCTCAAGTTCA	337	GGATATGGGCCTTGATATTCATG	436	100

Additional Table 3. Sequencing results of PCR products from primers of genes designed for this experiment. Best hits using BLASTN (http://www.ncbi.nlm.nih.gov) are shown.

Gene	SEQUENCE
ACADSB	GGCCTGTCTTCCGTGAACTCCTTCCTCATGCCCTTAATTTCTTTCTCAGGTCAGGAAGGCCTTCAGGCTTCAAAGGCGGACAAAAAAT
ACAT1	ACGTAGTAGGTTCATAGTAGGTCTATAAGAACACCCCATGTGATCCTTTCTAGGCAGTCTTTCCTCTCAA
AKT3	GCACGGTTCCTCGGAGGCCGCCACAGGTTTTCTATGGTGCAGAAATTTGTCCTCCTGCCCTTTGGACTATCCTACATACGCNCNTTAAG
CLDN1	CGCATGTGCGCGAGAGCACAGAAGAAAGTCCGGAAGTGGCCGTGCTCTTTGGGGGCCGGTAATCTTTCTTATTTCAGGGTCCTGGCAGACC
CLDN4	AGCAGGTACCTTTTAATTTTTTTCTTTCGTCTTTGTTTCTCCCGTGGACTGGAGCTCACTGCAATCTGTGAAA
CPT1A	CGTGGGGGGAGGAACATCTCCTCGAGGGAGAGGGCCGCAGTATGGTTGAACAGCAACTACTTCGCGAACCTTTTAGAGGCGTAT
DSG1	GAATATATCGCACTTGTCTTGGTGTTAGAAATAAAGGCTGGAATTTCATCAAATCAATTATGGTCTCAATATAAACTCACAGGCAACCTGCAATAAA
EGFR	GGTACAGCCAAAAGGAACAATCCCCCGAATTTGAGTCCTTAAGGAAGTATATCCAAGGTCCTTTGGAGGTCCAA
EREG	GGGCATGTGGGGCAGATTTTTTATTTTTCTGAGTCCGTGAGAGAGGATTAGTTAAATTTCAACCTGCCCCTGAGGTGTCAA
GAPDH	GGGCGTCAGTCCCTACGACACCTTGTTCAGCTCATTTCCTGGTACGACAATGGAATTTGGCCTACAGGCAACAGGGTA
HMGCL	CGCTTAGGGAAACTTCCCGGGCTAAGTCGCTGAGGTCACCAAGAAGCTGGTACTCCCATGGGCTGCTACGAGGAA
HMGCS2	GTCGCTGTGCAGGTCAGCTCTCGGCGTCCCGCTGGAGAGCTGGTATCGCAGTACATCGAGACCGTGCAGAAACTTGCTGCCAAACCAGAA
IGFBP5	GGGGTGTTAATGGAGGTGGGGTGCATATTTTCACTTCCCCATTGCAGGCCCTCTGAGAAATCAATCCACACCTTCTGAATAGGTAATA
LDHA	TGCGACTAGTGGCTGTGAGGATAAGTGGGCTTTCCCAAAAACCGGTGGTTAATTGGGAAGGTGGGTTGGCAATCTGGGAATTCAGCTCGCTTTCCAA
NQO1	GCATGATCGTAGGACCCGGGACTTTCAGTATCCTGGCCGAGACTGGTTTTAGCTTATAAAGAAGGCCGTCTCGAAA
PFKL	GGCGGCGTGGATCGGGAGTAGAGGTGCCACGGAGCACTACACCACAGAGGTTTCCTGGTACAACCTCTACTCCTCCAGATTTTTTTGGGGGGG
SLC14A1	CGCACCTTACTCGGTGTTGAGCAAATGGGGACCCTGCCCTGTCTTTCACTCCTGCCCCTTTCAATATGGCGGTTGTCAATGGTACAAAAA
UXT	GTCTAAACGTCCCCTCCGGGGGCTCAGCGACAACCTTCACCAAGGGACTTCCATTGAATATTCAAGGGCCCATTATTCCAA