Transcriptomic and metagenomic impacts of dietary energy of milk replacer in pre-weaned Holstein heifers

Connor Owens

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

Master of Science
In
Dairy Science

Rebecca Cockrum
Kristy Daniels
Alan Ealy

May 5, 2017
Blacksburg, VA

Keywords: Dairy, Calf management, Rumen microbiome, Transcriptome, Growth and development.

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ACADEMIC ABSTRACT

The variability in calf management can change the physiological state of the calf as they are weaned or attain puberty. It is up to the producer to ensure that the calves develop properly to meet their expected needs on the farm. While there are guidelines from the NRC in place, there is a substantial range in the amount of protein and fat that a calf can be fed. This physiological state can be reflected in the proteins produced in tissues, the expression of gene regulatory pathways, or even the microbes present in the gut. The purpose of this study was to examine how an increase in dietary energy in milk replacer of pre-weaned Holstein heifers impacts the microbial profile of the rumen as well as the transcriptome in tissues related to growth and metabolism. Our hypothesis was that pre-weaned Holstein heifers on milk replacer diets with lower dietary energy will have a different rumen microbiome composition and a different transcriptome in growth related tissues.

Holstein heifer calves (n = 36) were assigned randomly to 1 of 2 milk replacer diets: restricted (R; 20.9% CP, 19.8% Fat; n = 18) or enhanced (E; 28.9% CP, 26.2% Fat; n = 18). Calves were euthanized and rumen fluid was collected at pre-weaning (8 wks; n = 6) or post-weaning (10 wks; n = 6). Liver (L), adipose (A), and longissimus dorsi (LD) tissues were collected at pre-weaning (8 wks; n = 12). Average daily gain (ADG) and gain-to-feed ratio (G:F) were calculated for each calf. Analysis of ADG and G:F was

performed using a PROC GLM in SAS with diet as the main effect; E calves had increased ADG and G:F compared to R calves.

For rumen samples, libraries were constructed from extracted DNA and DNASeq was conducted using a paired-end analysis at 100 bp using Illumina HiSeq 2500. Operational taxonomic unit (OTU) clustering analysis was conducted using the 16s rRNA Greengenes reference. A PERMANOVA analysis was conducted in R to determine OTU populations for age and treatment. There was no difference in microbiome composition between pre-weaning and post-weaning calves ($P = 0.761$). Microbiome composition differed between E and R calves ($P < 0.001$). Bacteroidetes and Firmicutes represented the most abundant phyla for both E and R calves. Enhanced calves had 49.4% (5141 reads) Bacteriodetes and 36.4% (3789 reads) Firmicutes; whereas, R calves had 31.6% (2491 reads) Bacteriodetes and 41.1% (3236 reads) Firmicutes.

For L, A, and LD samples, libraries were constructed from extracted RNA for RNA-Seq analyses. RNA-Seq analysis was performed using CLC Genomics Workbench and the Robinson and Smith Exact Test was used to identify differentially expressed genes between diets. There were 238 differentially expressed genes in A, 227 in LD, and 40 in L. Of the differentially expressed genes, 10 appeared in at least 2 tissues. PANTHER was used to identify functional categories of differentially expressed genes. The majority of genes were associated with metabolic processes (A = 112, 26.7%; L = 16, 32.0%; LD = 81, 34.0%) or cellular processes (A = 93, 22.1%; L = 13, 26.0%; LD = 73, 30.7%). In E calves, upregulated genes included those regulating NADH

dehydrogenation (LD = 17, A = 5; i.e. *ND1*, *ND4*), gluconeogenesis (LD = 2, A = 6; i.e. *ALDOB, PCK2*), and cell proliferation (LD = 2, A = 3; i.e. *GADD45A, CDKN1A*).

There was a difference in both the transcriptome and rumen microbiome of calves fed differing levels of dietary energy. The calves on the R diet had a rumen microbial composition more similar to a younger calf, while the composition of E calves was more similar to a mature calf. The change in regulation of genes involved in the cell cycle and ATP synthesis in response to dietary energy could explain the change in ADG between diets. Because the R calves appeared to have stunted development of their microbiomes and an expression profile similar to oxidative stress, it is possible that the R diet did not meet the nutritional requirements of that calves.

GENERAL ABSTRACT

Changes in the way a calf is raised from birth can affect the biological processes that occur when they change from liquid to solid feed or reach reproductive maturity. While there are guidelines in place in how much a calf should be fed, there is still a large range in the amount of protein and fat in the liquid feed. The change in nutrition levels changes the biological processes occurring in the calf, which are reflect by changes in expression of genes in different parts of the calf as well the levels of microbes in the gut. The purpose of this study was to examine how the change in protein and fat in the liquid feed of female calves affects the microbes in the first section of the stomach, the rumen, as well as the genes expressed in parts of the calf associated with growth. Our hypothesis was that female calves fed liquid diets with lower protein and fat will have different rumen microbes and a different level of gene expression in growth related tissues. Female calves (n = 36) were randomly assigned 1 of 2 diets at birth: restricted (R; 20.9% Crude Protein, 19.8% Fat; n = 18) or enhanced (E; 28.9% Crude Protein, 26.2% Fat; n = 18). Calves were euthanized and rumen contents were collected at removal of the liquid feed (8 wks; n = 6) or 2 wks after calves were switched to an all dry feed diet (10 wks; n = 6). Liver (L), adipose (A), and longissimus dorsi (LD) tissues were collected at removal of the liquid feed (8 wks; n = 12). Bacterial DNA was extracted from the rumen samples and RNA was extracted from L, A, and LD samples. DNA and RNA were sequenced at the University of Missouri DNA Core Lab. Microbiome composition differed between E and R calves ($P < 0.001$). Enhanced calves had 49.4% Bacteriodetes and 36.4% Firmicutes; whereas, R calves had 31.6% Bacteriodetes and 41.1% Firmicutes. There were 238 differentially expressed genes in A, 227 in LD, and 40 in L. Of the

differentially expressed genes, 10 appeared in at least 2 tissues. In E calves, upregulated genes included those regulating NADH dehydrogenation (LD = 17, A = 5; i.e. *ND1, ND4*), gluconeogenesis (LD = 2, A = 6; i.e. *ALDOB, PCK2*), and cell growth (LD = 2, A = 3; i.e. *GADD45A, CDKN1A*). There was a difference in both the gene expression and rumen microbiome of calves fed differing levels of protein and fat. The calves on the R diet had a rumen microbial composition more similar to a younger calf, while the composition of E calves was more similar to a mature calf. Because the R calves appeared to have stunted development of their microbiomes and an expression profile similar to oxidative stress, it is possible that the R diet did not meet the nutritional requirements of that calves.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Rebecca Cockrum, for always giving me the support whenever I needed during the duration of my degree, but also pushing me to make difficult decisions when it came to my research. My committee also provided great insight and forethought when planning my project, and for that I say thank you. I would also like to thank my family for allowing me to pursue a career in agriculture and research, even if I did not go to UVA. Thank you to all of the graduate students in the Dairy Science and Animal Science department who provided insight and feedback when I was preparing for presentations and conferences. Thank you to the staff at Sharkey's for keeping me grounded and organized in planning my research. Finally, I would like to thank Gunter for giving me something to look forward to after a long day in the office. Thank you to Land O'Lakes Inc. (St. Paul, MN) and Tom Earleywine for providing milk replacer and discussions during the trial. We also thank the Virginia Tech farm crew and veterinary staff for assistance throughout the trial. We also acknowledge grant support from the USDA Agricultural and Food Research Initiative (AFRI) competitive grants program, no. 2016-67015-24565 "Impact of Pre-weaning Nutrition on Endocrine Induction of Mammary Development in Dairy Heifers" awarded to R. M. Akers and USDA award no. 2016-67011-24703, a pre-doctoral fellowship awarded to A. J. Geiger from the USDA-Food, Agriculture, Natural Resources and Human Sciences Education and Literacy Initiative competitive grants program.

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LIST OF ABBREVIATIONS

ADG – Average daily gain
AGE – Advanced glycation end product
ATP – adenosine tri-phosphate
BW – body weight
CP – crude protein
DISC - death-inducing signaling complex
DM – Dry matter
E – Enhanced diet (28% CP, 25% Fat)
EGF - epidermal growth factor
EST – expressed sequence tag
FADD - FAS-associated death domain
$FADH_2$ - Flavin adenine dinucleotide
G:F – Gain to feed ratio
G1-CDK - cyclin dependent kinases during the G1 phase
HPG - hypothalamic-pituitary-gonadal
KEGG – Kyoto Encyclopedia of Genes and Genomes
LD – Longissimus dorsi
MAPK - mitogen-activated protein kinase
NADH - Nicotinamide adenine dinucleotide
NRC – National Research Council
OTU – Operational Taxonomic Unit
PANTHER - Protein Analysis Through Evolutionary Relationships
PDGF - platelet-derived growth factors
QTL – quantitative trait loci
R – Restricted diet (20% CP, 20% Fat)
RFI – Residual feed intake
RIN – RNA integrity number
RNA-Seq – RNA sequencing
RPKM - reads per kilobase of transcript per million reads mapped
SNP – single nucleotide polymorphisms
TNF – Tumor necrosis factors
WGS - whole genome shotgun sequencing
VFA – volatile fatty acid

INTRODUCTION

Across the United States, variability in calf management changes the physiological state (i.e. growth rate, health, metabolism) of the calf (USDA-APHIS, 2016). The calf can be affected by how they are housed, the environment they are raised in, the type of diet they are consuming, and the source of the energy of their diet. While the National Research Council (**NRC**) has established the nutritional requirements for dairy cattle, there is still a large range within these requirements (NRC, 2001). A producer's ideal management style will vary depending on the goals of their farm. While the specific goals of some farms vary, producers want to have their calves weaned as soon as possible. Solid feed diets are less expensive than liquid feed diets and require less intensive labor during feeding (Jones, 2013). Weaning at 4 wks rather than 8 wks of age reduces the time liquid feed is used and will save the producer up to $55 per calf (Jones, 2013).

Before a calf is weaned, its rumen must be sufficiently developed such that the calf can sustain its nutrient needs from a diet of 100% solid feed. This transition from non-ruminant to ruminant metabolism is usually estimated using the intake level of the calf, their age, as well as their weight. In the US, approximately 50.2 ± 1.8% of all farms use age as the main criteria for weaning, while only 21.5 ± 1.5% use calves eating 0.91 kg or more of starter for 3 consecutive days (USDA-APHIS, 2016). But some indicators of maturity may be seen at a smaller scale. To digest plant material efficiently, a calf should have a rumen microbial composition similar to an adult at the time of weaning. The microbial profile of the rumen starts to be more similar to a mature cow at 3 wks of age, but it is not until 4 to 5 wks of age until the calf will voluntarily eat enough starter to

be safely weaned (Jami et al., 2013; Rey et al., 2014; Jones, 2013). The rumen microbial profile could possibly be used a method to assess if a calf is ready to be weaned. While there have been studies examining how diet affects the rumen microbial population in mature cows, there have not been many similar studies conducted in calves.

Variation in management could also affect the expression level of certain genes in the calf. The transcriptome, or the range of expression of all mRNAs in an organism, changes based on factors such as age, environment, or physiologic state of the calf (Adams, 2008). There is also a variation in the transcriptome in the calf depending on the tissue being examined. Changes in the diet of calves could affect the transcriptome in tissues such as portions of the GI tract, tissues more closely related to growth, or tissues more closely related to metabolism. As the calf grows, they should upregulate genes related to gluconeogenesis in the liver while glycolysis related genes should be down regulated in skeletal muscle (Howarth et al., 1968). Calves placed on diets with differing levels of energy would be expected to grow at different rates, which could lead to the differential expression of genes related to gluconeogenesis and glycolysis.

The purpose of this study was to examine the effects of varying dietary energy from milk replacer on the rumen microbiome and the transcriptome in tissues related to growth and metabolism in pre-weaned Holstein heifers. Our hypothesis is that there will be a difference in the rumen microbial composition and the transcriptome in tissues related to growth and metabolism. Calves fed a higher level of dietary energy should have more diversity in their rumen microbiome. Genes related to metabolism are expected to be up-regulated in calves fed a higher level of dietary energy.

CHAPTER 1

LITERATURE REVIEW

1. Calf Management

Dairy calf management can vary greatly from farm to farm. This can be attributed to colostrum management, differences in housing, number of feeding times, amount fed, and feed composition (USDA-APHIS, 2010). There is a large variation between dairies with type of diet given to pre-weaned calves; 57.5 ± 1.4% of farms provide medicated milk replacer, 30.6 ± 1.3% unpasteurized waste milk, and 28.0 ± 1.3 unpasteurized whole/saleable milk (USDA-APHIS, 2010). Certain changes can be more beneficial for the producer, as they require less labor or time devoted to the calves, while other factors have a greater impact on the long-term health and performance of the calf. Specifically, changes in the level of nutrients in the milk replacer can affect milk production. Calves that were able to consume more nutrients prior to 56 d produced 1,000 – 3,000 more lbs of milk than calves that were placed on more restricted diets (Soberon et al., 2012). Management and nutrition of the calf will shape the calf's health, growth, and future productivity. The most important factors are the amount of money and time a producer is willing to invest in their calves in order to make a profit in the future.

1.1 *Preweaning Management*

When calving occurs, the newborn calves should be fed 2.84 – 3.79 L of colostrum, within the first hour of life (BAMN, 2003). Whether or not a calf ingests the colostrum can affect their innate immune response and overall survivability (Raboisson et al., 2016). The management of intake of colostrum is just the first of many decisions a producer has to make for their pre-weaned calves. The producer will then have to decide

what the calves will be fed the during the pre-weaned period, when starter is first given, and when a calf is weaned.

1.1.1 Amount of Milk Replacer

After ingestion of colostrum ceases (24 - 48 hrs after birth), dairy calves are fed milk or, more commonly, milk replacer to meet nutritional requirements until weaning. Usually in the US, a calf is fed mixed milk replacer (solids and water) at 10% of their body weight (**BW**) per day (Lorenz, 2011). At this level of nutrition, calves are undergoing "restricted feeding" where the nutrients are mostly meeting maintenance requirements and little weight gain occurs (Lorenz, 2011). The primary rationale behind this style of management is economic; whether this comes from less time and labor spent feeding or less milk replacer used for each calf. In the past, milk replacer has been cheaper than feeding whole milk. But with the recent increase in whey protein used in milk replacer, milk replacer is slightly more expensive than whole milk (Jones and Heinrichs, 2017; Table 1.2).

While feeding at 10% BW is common practice, calves offered *ad libitium* from the teat, tended to ingest about 20% of their BW/d, or 10 – 12 L of milk (Jasper and Weary, 2002). Calves placed on restricted feeding programs only gain 20-30% of their potential growth. This impairment in nutrition and growth can negatively affect immune response (Woodward, 1998; Appleby et al., 2001). Calves fed 15% of BW will exceed 50% of their growth capacity in moderate weather conditions (NRC, 2001). This also allows for producers to still be able to feed calves twice a day without going over the capacity of the abomasum, which is about 2 L at birth and 6 L at 8 wks (Schmidt and Zsedely, 2011).

1.1.2 Energy Sources for Calf Milk Replacer

While the most fat used for milk replacer is derived from animal fat sources, the source of protein in milk replacer varies greatly (Akey, 2007). Most protein used originates from all-milk sources, such as dried whey protein, skim milk, or sodium/calcium caseniate (BAMN, 2014). Since the 1950s, milk whey protein has been the predominant choice, as it has provided a more cost-efficient option than feeding whole milk (Table 1.3). Recently, the price of whey protein has increased 250% per year (Jones and Heinrich, 2017). As this price continues to increase, alternative sources of proteins have been investigated for their nutritional content. Non-milk based proteins include soy, wheat, egg, blood plasma, and potato (Jones and Heinrich, 2017). While cheaper, these non-milk proteins are not directly equivalent to milk proteins.

1.1.3 Levels of Energy of Milk Replacer

While the quantity of liquid feed being fed is important, the composition and quality are just as important, if not more so. Residual or waste milk can be used, but this leads to multiple health issues with the calves. Milk could transmit infectious pathogens that would compromise the health of the calf. Waste milk can contain pathogenic levels of bacterial residues such as *Streptococcus, Enterobacteriaceae,* and *Staphylococcus* (Selim and Cullor, 1997). Milk that is pasteurized before being fed to the calves leads to higher costs in both labor and equipment (Godden et al., 2005). Therefore, milk replacer is most commonly fed to calves before weaning to ensure that the liquid feed is free of pathogens, is consistent in composition, and to forgo the higher costs.

The type and composition of milk replacer varies from farm to farm depending on the goals and budget of the farm. The most important variables in milk replacer are fat

and crude protein (**CP**). Having the optimal level of CP in milk replacer is essential for providing amino acids for tissue synthesis, while having the ideal level of fat is necessary for providing energy used in maintenance, growth, and development (USDA-APHIS, 2008). Protein levels in milk replacers can range from 18% to 30%, with most formulas falling between 20% to 22%, while fat levels have a greater range of 10% to 28%, with most formulas containing 18% to 22% (USDA-APHIS, 2008). The level of fat and protein as well as the protein to energy ratio are important factors contributing to calf growth and performance, even possibly impacting the future performance of the cow.

1.2 *Postweaning Management*

Transitioning a calf from an all liquid to an all solid diet requires proper planning to ensure that the rumen is adequately developed and can digest solid feeds. Calves must be provided starter early enough to physically stimulate rumen papillae development without taking up too much space in the rumen and abomasum. The time of weaning is not directly determined by the calf's age, but rather by their size and growth rate. Age can be used as a proxy measurement, but the size and growth rate still need to be taken into consideration when choosing a time to wean the calves.

1.2.1 Time of Weaning

From a strictly economic standpoint, because liquid feeds are more expensive than solid feeds such as starter, calves are weaned off liquid feeds as soon as possible. In the United States, 70% of operations wean at around 7 weeks of age, while 25% tend to wean at around 9 weeks of age (Jones and Heinrichs, 2007). The primary reason for weaning calves at a younger age is to start rumen development earlier. Similar to level of fat and protein in milk replacer, the time of weaning varies depending on size of the herd.

Smaller farms (< 30 cows) wean their calves around 11.6 ± 0.9 weeks, while moderate herds (> 500 cows) wean their calves at 8.9 ± 0.1 weeks (USDA-APHIS, 2016) (**Table 1.3**). At 11 wks, heifers weigh approximately 117.94 kg, while 9 wk heifers weigh 86.64 kg (USDA-APHIS, 2010). On average, calves are completely weaned when they consume 0.91 kg of starter per day for 3 consecutive days, which usually occurs between 6 and 8 wk of age (Jones, 2013). Calf age, weight, and level of starter intake all influence time of weaning. The factor used to make the decision varies by herd size (Table 1.4).

If the calf is weaned too early or too late, they can incur unnecessary costs. Calves can be weaned as early as 3 wk of age, but these calves will not voluntarily eat the starter given and require more management to stimulate starter intake (Kehoe et al., 2007). While calves weaned at 3 wk do have the same growth measurements as those weaned at 8 wk, calves weaned at 4, 5, and 6 wk also showed no change in growth measurement and required less management (Kehoe et al., 2007). If the calf is weaned too late, money could be wasted on milk replacer and slowing rumen development.

1.2.2 Starter

While time of weaning influences cost for the producer, it is also important to examine the composition of the starter given at time of weaning. Starter is initially offered around 1 wk of age to stimulate development of the rumen (NRC, 2001). Calves will initially nibble on the starter for the first 2 wks, with a large increase in intake occurring between 3 to 4 wks of age (Amaral-Phillips et al., 2006). Starter should contain ingredients that meet the nutritional needs of the calf, but are also palatable. The current recommended starter should contain 16 – 20% CP, 0.70% calcium, 0.45% phosphorus, 0.65% potassium, and various levels of vitamins and minerals (NRC, 2001). A typical

starter with 18% CP should contain ingredients such as oats (rolled or coarse ground), cracked corn, soybean meal, molasses, white salt, dicalcium phosphate, and limestone (Amaral-Phillips et al., 2006).

2. Rumen Development

When a calf is born, the first three portions of the stomach (the rumen, reticulum, and omasum) are not well developed, as they are not needed for digestion of liquid feed. With the small intake of starter at early weeks of age before weaning, the rumen is stimulated to grow and develop features needed for digestion, such as papillae (Brownlee, 1956); Sander et al., 1959; Anderson et al., 1987). By the time the calf is weaned, the rumen should be developed enough to physically hold what the calf ingests and to chemically digest efficiently. However, the majority of feed breakdown is performed by the microbes present in the rumen; therefore, the microbial profile at weaning should be well developed for solid feed digestion.

2.1 *Microbiome Development*

Calves need to have the right proportion of bacteria to efficiently breakdown feed in the rumen. The intake of non-solid food (milk or milk replacer) within the first 24 hrs is one of the first influences establishing the rumen microbiome (Rey et al., 2014). The microbiome is then reshaped after the calf begins to consume solid foods such as starter at 15 d (Rey et al., 2014). Microbes present in a dairy cow's rumen are responsible for conversion of plant-based feed to energy. One such way is through the production of volatile fatty acids (**VFAs**, i.e. acetate, propionate, butyrate) in the rumen, which contribute to approximately 70% of the caloric requirements (Bergman, 1990). At 1-3 d of age, most of the microbes present in the rumen are either from the phylum

Proteobacteria (45%) or *Firmicutes* (30%; Jami et al., 2013). A major shift in the microbiome takes place on d 3. Around d 3 the level of *Proteobacteria* drops from 70.4% to 16.9%, the phylum *Bacteriodetes* increases in from 13.9% to 56.3%, and the level of *Firmicutes* remains the same at 13.9% (Rey et al., 2014). On d 3, those associated with *Streptococcus* dropped to near 0% and the level of *Proteobacteria* increased to about 50% (Jami et al., 2013).

2.1.1 Normal bacterial composition

Proteobacteria are gram-negative bacteria primarily composed of pathogens such as *Escherichia coli*, *Campylobacter concisus*, or those in genus *Vibrio* (Mukhopadhya et al., 2012). They are also thought to be responsible for nitrogen fixation as well as having properties that exploit a host's immune system and cause proinflammatory change (Mukhopadhya et al., 2012). While the mammalian gut microbial community is relatively stable on its own, diseases associated with metabolism and immune response often lead to an imbalanced gut with and an increase in *Proteobacteria* in more mature mammals (Shin et al., 2015).

While *Firmicutes* are found in other regions of the digestive tract, the major families found in the rumen are unclassified *Ruminococceae*, *Rikenellaceae*, and *Christensenellaceae*, implicating that that the *Firmicutes* in the rumen are primarily responsible for the starch and fiber digestion (Mao et al., 2015).

Bacteriodetes is the predominant phylum in the rumen of healthy dairy cows for the remainder of their life, with the genus *Prevotella* accounting for 19.08% of the total reads present in a rumen bacteria sample (Rey et al, 2013; Jami et al. 2013). These bacteria are also thought of as being primarily responsible for fiber digestion because

they comprise the majority of microbes present in the bacteria, but no other portion of the GI tract (Mao et al., 2015).

The overall composition of the microbiota seems to remain stable until the removal or milk replacer/introduction of starter at 7 wks, where *Proteobacteria* is reduced even further in healthy calves and the microbial community is almost entirely *Bacteriodetes* or *Firmicutes* (Jami and Mizrahi, 2012). After the calf is weaned, their rumen microbiome remains relatively constant as long as they are on the same diet. Changes in diet, such as change in the level of forage or hay, can lead to a shift in the microbes in the rumen (Ellison et al., 2014).

2.1.2 Dietary effect on rumen microbiome population

Once the rumen microbial population has been established, post weaning, the population can shift depending what is being fed to the animal. Typically on a 30% forage and 70% concentrate diet, the Holstein-Friesian rumen is comprised of 51% *Bacteriodetes*, 42% *Firmicutes*, 5% *Proteobacteria*, and 2% other phyla (Jami and Mizrahi, 2012). The microbiome can reflect a change in forage to concentrate ratio. In sheep, an increase in forage leads to an increase in *Bacteriodetes* in the rumen, while an increase in concentrates leads to an increase in *Firmicutes* (Ellison et al., 2014). Petri et al. (2013) reported a high forage diet led to *Ruminococcus* (a genus within the phylum Firmicutes) comprising the largest portion of the rumen microbiome (8.01%), while a high grain diet caused the *Ruminococcus* to decrease to 5.70%. This change in diet also caused *Prevotella* (a genus within the phylum *Bacteriodetes*) to increase from 2.86% to 7.75%. Knowing the relationships between bacterial phyla with diet and feed to energy conversion can lead to developing strategies to select for feed efficiency.

Probiotics, or live microorganisms that can be incorporated into the feed, can be incorporated into the animal's feed to temporarily change the rumen microbiome. These probiotics can be given as a calf or during lactation to improve energy intake. Kmet et al. (1993) determined that dietary yeast in the diet of calves and lambs improved both their feed intake and their live weight gain. A large issue with these probiotics is that the results are extremely variable and are not permanent; in order to maintain the effect of the probiotic, it needs to be administered daily (Uyeno et al., 2015).

2.1.3 Genetic effect on rumen microbial population

One reason the microbiome reverts to its original composition after a probiotic is administered may be associated with underlying genetic mechanisms (Rowe, 2017). Because the gut microbiome varies between individual animals, it may be possible to transfer the gastrointestinal (GI) contents of one cow into another to influence the microbial population (Weimer et al., 2010). Using an automated ribosomal intergenic spacer analysis (ARISA) to "fingerprint" the rumen microbiota, pairs of cows were selected based on different microbial population, pH, and VFA content and had ≥ 95% of their rumen contents switched. While the pH and VFA content of the rumen was restored to pre-exchange levels within 48 hrs, the microbial content of each animal was restored to pre-exchange profiles after about 2 wks (Weimer et al., 2010). While it is possible to change the microbial population of the rumen, the effect is only temporary. Though the microbiome varies between individuals, there are common similarities between the rumen contents of the same species. While there is a large variety of bacteria present in the rumen, it is possible to identify all bacteria present in the bovine rumen with as little as 10 samples (Jami and Mizrahi, 2012). There was a 51% similarity between all of the

samples. Also, there were 32 shared genera between the samples with varying abundances, suggesting that there could be a core group of microbes present in every rumen whose abundance help shape the microbial profile of the rumen (Jami and Mizrahi, 2012).

In multiple studies, there has been variation between individual animals in the microbial population of the rumen, resulting in a relationship with feed efficiency and methane emissions (Roehe et al., 2016). There was a difference in methane emission among sire progeny groups, as well as a similar ranking of rumen microbes when methane emissions is expressed as per day or per DMI. This indicates that a direct genetic influence of the host on the rumen microbial methane production could exist independent of what is consumed (Roehe et al., 2016). In mice, 18 host quantitative trait loci (**QTL**) were linked to relative abundances of microbial taxa within the gut (Benson et al., 2010). There has been the linkage of specific taxa with certain single nucleotide polymorphisms (**SNP**s) in humans. *Actinobacteria* and *Bifidobacterium* were significantly linked to the SNP rs651821 on the *apolipoprotein A-V* gene (*APOA5*; Lim et al., 2016). If the host has influence over the rumen microbes associated with traits such as higher feed efficiency or lower methane emissions, the microbial profile could be used as a method to select for these traits in dairy cattle.

2.2 *Gene pathways involved in growth of calves*

2.2.1 Gluconeogenesis

As the calf develops and matures, the expression of gene regulatory pathways vary. These can be in relation to growth, metabolism, response to the environment, or any combination thereof. As the calf develops and grows, the mechanism for converting feed

into energy changes based on the diet of the calf. There are changes in tissues related to growth and metabolism that occur as the rumen develops (Howarth et al., 1968). The liver decreases its level of oxidized glycogen and increases level of gluconeogenesis. Skeletal muscle reduces activity of glycogen synthesis pathways. Adipose tissue increases its capacity to synthesize fatty acids (Howarth et al., 1968). In sheep, enzymes related to gluconeogenesis coincide with the change in use of the rumen. Glucose-5-phosphotase and fructose-1,6-bisphosphotase activity increases with the development of the rumen, while pyruvate carboxylase and lactate dehydrogenase decrease in activity (Baldwin, 1998). Pyruvate carboxylase is an important rate-limiting step for gluconeogenesis, so any change in regulation to genes controlling this activation could impact the ability for the calf to generate ATP through this pathway (Greenfield et al., 2000). While this change in activity is not associated with growth, it could play an important role in identifying the level of gluconeogenesis that is occurring in calves. Lower dietary energy to the point of starvation in yaks caused an increase in expression of pyruvate carboxylase (Yu et al., 2016). Dairy calves could have a similar expression level of pyruvate carboxylase during the intake of lower dietary energy.

2.2.2 Oxidative Phosphorylation

The mitochondria produce ATP through 1 of 2 pathways: 1) the citric acid cycle or 2) oxidative phosphorylation. While both are efficient in producing ATP for the cells to use as a source of energy, oxidative phosphorylation is responsible for 80% of the ATP produced by the mitochondria (Dean, 2010). Oxidative phosphorylation is the process along the mitochondrial membrane where ATP is synthesized as a result of the transfer of electrons from NADH or $FADH_2$ to O_2 (Berg et al., 2002). These NADH and $FADH_2$

molecules are the product of other metabolic processes, such as the citric acid cycle, glycolysis, or fatty acid oxidation (Berg et al., 2002). The pathway of oxidative phosphorylation involves 5 units in the membrane of the mitochondria: 1) NADH dehydrogenase, 2) succinate dehydrogenase, 3) cytochrome bc1 complex, 4) cytochrome c complex, and 5) ATP synthase (KEGG, 2014). Specifically, genes controlling NADH dehydrogenase, such as the MNTD group of genes, can affect ATP generation ability of an organism (Wang et al., 2008). These genes remove the hydrogen ion from NADH within the mitochondria, which creates a proton gradient at the mitochondrial membrane. This gradient drives the other 4 complexes involved in oxidative phosphorylation. These complexes need to function properly in order to efficiently produce ATP (Freeman, 2002). If there is an effect on the NADH dehydrogenase complex that causes a smaller proton gradient to form, less ATP would be generated from the ATP synthase complex.

2.2.3 Cell proliferation and apoptosis

As the calf matures, tissues such as skeletal muscle and liver are growing to support the added weight and increased metabolism. The mechanisms controlling this growth vary depending on the tissue. In general, cell cycle genes and external factors impact cell proliferation (Breier and Gluckman , 1991). Some of these external factors are mitogens, which are extracellular substances that regulate cell proliferation (Alberts et al., 2002). These can include growth factors, such as platelet-derived growth factors (**PDGF**) or epidermal growth factor (**EGF**), and stimulate the expression of pathways, such as the mitogen-activated protein kinase (**MAPK)** pathway. The MAPK pathway increases production of Myc, a gene regulatory protein that increases the transcription of genes that increase the activity of cyclin dependent kinases during the G1 phase (G1-

CDK) (Alberts et al., 2002). If any genes related to the MAPK pathway are highly prevalent in a tissue, then the tissue has enough energy to grow and proliferate. Muscle cells develop through either hypertrophy or hyperplasia. These pathways are regulated by insulin like growth factor 1 (*IGF1*), phosphatidylinositol-3 kinase (*PI3K*), and protein kinase B, or Akt (Egerman and Glass, 2014). Up-regulation of genes involved in these pathways signals that the muscle is growing in mass (Egerman and Glass, 2014).

The overexpression of proteins such as Myc is associated with cancer, as continuous proliferations of cells can lead to the formation of tumors (Alberts et al., 2002). While this may be true in some tissues, cells also have a mechanism to control excess proliferation. This mechanism of programmed cell death is referred to as apoptosis. The extrinsic pathways controlling apoptosis involve transmembrane receptors, particularly tumor necrosis factors (***TNF)*** (Locksley et al., 2001). Through a cascade of binding of the FAS-associated death domain (*FADD*) protein and *TNF* ligand to the *TNF* receptor, the death-inducing signaling complex (DISC) forms and triggers the execution phase of apoptosis (Kischkel et al., 1995). There is also an intrinsic mechanism controlling apoptosis. There are non-receptor-mediated stimuli that affect certain targets within in the cell and are usually initiated by the mitochondria within the cell (Elmore, 2007). The stimuli can act as a positive response (e.g., toxins, hypoxia, hyperthermia, or viral infections) or negative fashion (e.g., the absence of a certain hormone, growth factor, or cytokine that suppressed apoptosis pathways; Elmore, 2007). While apoptosis can be initiated by the cell itself through the production and binding of certain ligands to receptors, health and environmental factors can also induce these apoptosis pathways.

3. Next-generation sequencing

3.1 *Metagenomic Analysis*

Ruminants cannot efficiently obtain the energy from plant-based feed on their own, so instead they rely on a variety of microorganisms within their rumen to assist with digestion. Because ruminants mostly consume plant materials as sources of energy, the microorganisms need to efficiently breakdown tough, complex compounds such as cellulose to obtain energy from the feed. This symbiotic relationship, where the microorganism provides energy for the ruminant and in return the ruminant provides a safe environment for the microorganism to thrive, is due to a series of complex relationships and interactions between the animals and the organisms. In order to properly study this interaction several sequencing and analysis methods have been developed and are continuously being improved upon. The genomic analysis of microbial DNA that is extracted directly from communities in environmental samples and how that genomic material interacts with its environment is called metagenomics, and in this case, the environment refers to the rumen itself (Nature, 2004). In the dairy industry, metagenomics has been used to identify the microorganisms responsible for diseases such as mastitis and endometritis (Oikonomou et al., 2012; Keuhn et al., 2013; Santos et al., 2011).

3.1.1 Sequencing Methods

There are two major methods used to study the metagenomes of animals: 1) whole genome sequencing and 2) 16s rRNA amplification. Both of these methods provide information on the bacterial composition of the rumen. Currently, that is the aim of most rumen metagenomics experiments. But, as the rumen microbiome becomes better

characterized, whole genome sequencing can provide additional information into the functions of the bacteria.

3.1.1.1 Whole Genome Sequencing

One method of sequencing that provides the largest amount of information is whole genome shotgun sequencing (**WGS**). This method takes into account the genetic sequences from all cells present in a sample, which usually contains cells from multiple microorganisms, animals, and plants depending on the sample. First, total DNA is extracted from the sample containing microorganisms, which is then subsequently sheared into fragments, the size of which is determined beforehand (Sharpton, 2014). The larger the size of the fragments, the more likely that fragment will be aligned to the correct position on the genome. But, if the fragment is too large, it becomes difficult to have high sequencing depth, or the number of reads that include a specific nucleotide at a specific location (Illumina, 2017). These fragments are then sequenced and aligned to a reference genome, which, for metagenomic sequencing, includes a large set of genomes from microbes, such as bacteria and archaea, and non-microbes, such as animals and plants (Sharpton, 2014). In dairy cattle rumen samples, WGS results in only 3.46 ± 2.19% of the sequences being aligned (Ross et al., 2012). This is mostly due to certain sequences from microorganisms that do not have a well-constructed reference genome. The level of similarity between the reference genome sequence being aligned can vary in percent match. Percent match refers to the percent the read matched the reference genome. The lower the percent match is to the reference, the more reads that will be aligned and assigned taxonomies. With the rumen, as well as most other metagenomic

studies, the standard percent match is 97%, which allows for some variance in the genome but not so much that the alignment is inaccurate.

3.1.2 Operational Taxonomic Unit Clustering

Once the bacterial DNA is sequenced, the reads are used to quantify the abundance of individual species. The reads are clustered together based on their similarity to one another. These clusters are referred to as operational taxonomic units, or OTU. The number of reads within each OTU represents the amount of that individual organism within the sample. The OTU are then compared between samples, groups, or in this case, dairy cattle. Using the analysis of these OTU clusters, researchers can compare what bacteria a present in the samples as well as analyze if any differences occur.

3.1.2.1 Advantages and disadvantages

Many of the limitations stem from the reference used for OTU clustering. While it is possible to adjust the accuracy required for a read to be mapped to a reference, the proper level for each type of sample is not always already well know. This often leads to over- or underestimation of the OTUs present . This also depends on the complexity and length of the sequences; the longer and more complex the sequences are, the higher the level of similarity should be (Chen et al., 2013). Two sequences from the same organism that only have a small difference that naturally occurs could be mapped to the different references if the level of similarity required is too high (Nguyen et al., 2016). This could lead to 2 reads from one organism being viewed as reads from 2 different organisms and subsequently clustered separately (Nguyen et al., 2016). One suggestion to reduce the amount of reads that might come across as white noise and lead to errors in estimation of a certain OTU is to filter the sequences before clustering begins by establishing a

taxonomic distance threshold (Schloss, 2010). This threshold would remove sequences that were too close during hierarchical clustering, but not similar enough to be clustered into the same OTU.

3.2 *RNA Sequencing*

Another important tool used in marker-assisted selection that can aid in understanding differential gene expression in dairy cattle is RNA sequencing (**RNA-seq**). In general, RNA-seq can characterize which transcripts are differentially expressed. By sequencing the mRNA, RNA-seq develops a transcriptome, or the set of transcripts expressed in a certain set of cells at a specific physiological state or point in development (Wang et al., 2009). The transcriptome can be used to functionally annotate the genome, or define what function(s) with which a gene is associated. In the RNA-seq protocol, mRNA is fragmented and converted into cDNA libraries. These cDNA libraries are sequenced and aligned to the reference genome. The amount of cDNA sequences aligned to a section of the genome is measured; this measurement represents the amount of mRNA expressed.

Before the RNA can be sequenced, the extracted RNA samples need to be prepared for sequencing. First, the mRNA is isolated from the total RNA sample. Usually mRNA only comprises 4% of the total RNA, so these need to be isolated by either isolating the mRNA or depletion of rRNA, which comprises 80% of the total RNA (Brown, 2002; NEB, 2017). The mRNA is then fragmented into approximately 200 nt long reads and then converted into cDNA. The cDNA is then converted into a molecular library with the ligation of adapters and is sequenced by a high-throughput sequencing machine, such as Illumina HiSeq 2500 machine (Mortazavi et al., 2008; NEB 2017).

While being sequenced, the cDNA fragments are turned into single-strand pieces and bind to the inside of flow channels. Unlabeled nucleotides are added and incorporated to the single strands. These are then denatured to leave single stranded template DNA. This repeats until there are millions of template DNA in each channel (Illumina, 2015).

Labeled nucleotides are then added to the channels along with primers and DNA polymerase in order for the nucleotides to be added to the templates. The fluorescence is captured via a laser that then records the specific nucleotide that has binded to single stranded DNA. This cycle repeats until the sequence for each fragment is recorded. These fragments are then aligned to the *Bos taurus* reference genome (Illumina, 2015). Each sequence is then classified into 3 separate categories: an exonic read, a junction read, or a poly(A) end-read. Using these categories, RNA-seq allows for the development of a base-resolution expression profile for each gene expressed (Wang et al, 2009). The expression of a gene is then measured using the number of total reads that fall into the exons of a gene and then normalized based on the length of unique exons that can be mapped (Wang et al., 2009). This expression level is measured using the number reads per kilobase of transcript per million reads mapped (**RPKM)**.

3.2.1 Transcriptomic Analysis

The sequence of an entire organism's DNA is called the genome. It provides information on what nucleotide bases are present and in what order along the DNA. But because the sequence of an organism does not usually change in their lifetime, a genome has little information to offer on its own in reference to that specific organism. While every cell in an organism contains the same genetic information, not every gene is active at the same time in every cell. This gene expression profile within each cell type is called

the transcriptome (Adams, 2008). The transcriptome represents the total amount of RNA that is part of extronic portions of the DNA, where transcripts that regulate cellular functions are created (Adams, 2008). By examining the amount of transcripts that are expressed at a certain time, the function of cells and amount of influence a factor has on the body can be examined.

3.2.2 Factors that affect the transcriptome

The transcriptome serves as a snapshot of the transcript expression levels at a given point in time. The changes in the transcriptome are caused by either change in age of the organism, physiological state, environment the organism grows in, or any other factor that affects the functions of the organism (Adams, 2008). Physiological state and environment have some of the larger effects on the transcriptome. State can refer to what state of maturity calves are in, how they are responding to stress, or how efficiently they are converting feed into energy. For example, during acute feed restriction, cows increased expression of genes associated with gluconeogenesis (*PC*, *PDK4*) and inflammation (*SAA3*) in the liver (Akbar et al., 2012). An example of environmental factors that affect the transcriptome is temperature of the environment. When cow is above or below a thermoneutral temperature (-15 °C to 25 °C), gene networks within and between cells and tissue types respond to the environmental heat to control cellular and whole-animal metabolism transcripts (Collier et al., 2008).

3.2.3 The transcriptome in relation to growth

The tissues primarily related to growth, such as skeletal muscle and adipose tissue, change the expression of genes depending on the rate of growth in the calf. Currently, the information on cattle growth transcriptome in muscle and adipose tissue

comes from beef cattle or bulls. While skeletal muscle contractile cells are expected to increase their expression of genes related to growth, they also focus on expressing genes to create an extracellular matrix to support the development of blood vessels and provide support for the muscle fibers (Relaix and Zammit, 2012; Paylor et al., 2011). In the longissimus dorsi of Brahman steers, the genes with the highest association with ADG/kg are those involved in the cell cycle process (n = 66; FDR Q-value = 2.58×10^{-5}) or extra cellular matrix development (n = 46; FDR Q-value = 1.24×10^{-4}; Guo et al., 2015). In postnatal calves, of the genes co-expressed with extracellular matrix development, there are about 27 involved in the cell cycle process and 30 involved in angiogenesis (Guo et al., 2015). These gene profiles are similar to those that provide scaffolding for adipose tissue developing at the same time in calves (Nakajima et al., 1998; De Jager et al., 2013; Guo et al., 2015). There is an upregulation of genes related to glycolysis in Charolais bull calves that had a high muscle growth rate at both 15 and 19 months of age (Bernard et al., 2007). There is a 0.77 genetic correlation between 5 genes related to cell cycle (*CDC6, CDC20, CDCA3, KIF20A, KIF23*) and ADG kg/d and a 0.70 correlation between 5 genes related to extracellular matrix development (*ADAMTS4*, *BGN*, *COL5A2*, *TGFB2*, *SERPINH1*) and ADG kg/d (Guo et al., 2015).

Adipose tissue is also important to growth and development of the animal, especially in the beef industry. Genes regulating adipogenesis and lipogenesis change substantially between birth and the early postweaning stage of development (Tan et al., 2005; Lehnert et al., 2007). These genes are responsible for creating ATP to be used for energy, which can be used for maintenance, growth, or invested back into metabolism. Another gene isotype that has gained more attention is PPAR, peroxisome proliferator-

activated receptors, which have been seen to be central controllers of metabolic coordination for an entire organism (Bionaz et al., 2013). The gene *PPARG* is highly expressed in adipose, rumen, and epithelial kidney cells called Madin-Darby bovine kidney cells, while *PPARA* is expressed more in the liver and kidney tissues (Bionaz et al., 2013). In order to fully understand how factors affect growth and metabolism in certain tissues, there needs to be a better characterization of these metabolic regulatory genes.

3.2.4 Advantages and drawbacks of RNA-Seq

There were methods before RNA-seq that allowed for transcriptome analysis, but the use of RNA-seq has brought forth new advantages as well as some drawbacks. Before RNA-seq, researchers used microarrays, cDNA/expressed sequence tag (EST) sequencing, and microsatellites as methods to identify transcript expression (Parkinson and Blaxter, 2009; Fortes et al., 2013). Since the incorporation of RNA-seq, the limitations surrounding these previous technologies have been surpassed. First, RNA-seq does not rely on using a previously determined genomic sequence. Another advantage RNA-seq presents is have single-base resolution during sequencing, as opposed to the several to 100 bp resolution of microarrays (Wang et al, 2009). This single base resolution is useful when examining how 2 exons are connected, which comes from 30 bp reads (Wang et al, 2009). This can also be used to characterize single nucleotide polymorphisms, or SNPs, in the transcription regions (Cloonan et al, 2008). Lastly, the amount of background noise from RNA-seq is extremely low because these DNA sequences can be mapped unambiguously to parts of the genome (Wang et al., 2009). There is no upper limit on the range of expression level with RNA-seq, as opposed to

DNA microarrays, which have a lower and upper expression limit of 100-fold to a few hundred-fold, respectively (Wang et al., 2009).

While RNA-seq requires relatively few steps and provides a wealth of information, there are challenges with the technology. Most sequencing methods require small fragments of the RNA (200 - 500 bp), but extracted RNA is larger to ensure that it does not degrade. RNA –Seq usually uses 75 – 150 bp fragments. This fragmentation can increase bias towards the ends of transcripts, but has little bias in the transcript body (Mortazavi et al., 2008). Another issue that might arise is that short artifacts with similar sequences to RNA reads could be amplified. This makes it hard to distinguish between a true RNA expression or PCR artefact presence without sequencing more samples (Wang et al, 2009). On a bioinformatics level, RNA-seq produces a problem with the output files. RNA sequencing can produce files that are very large and difficult to handle. The higher the quality and depth of sequencing, the larger the files created will be. For large transcriptomes, such as mammals, portions of the transcriptome can be mapped to multiple locations (Wang et al., 2009). The shorter the sequence, the higher the possibility of reads matched to multiple points on the genome can occur. It is possible to overcome this obstacle by proportionally aligning these reads based on the number of reads mapped to the surrounding unique sequences (Wang et al., 2009). Incorrect alignment resulting from these artifacts could increase the likelihood of Type I errors. Until these complications are corrected or an easier method for strand specific library construction is developed, transcriptome annotation will require a high level of RNA sequencing information.

4. Conclusion

Variations in milk replacer composition, specifically CP and fat content, change the level of dietary energy within the milk replacer. The industry standard milk replacer of 20% CP and 20% fat may not be an adequate reflection of what occurs naturally when a calf suckles directly from the dam's teat. The level of nutrition in humans can affect the gut microbiome, so variation in dietary energy could have an effect on the rumen microbiome in calves. The level of dietary energy also affects the ability for tissues such as muscle and fat to grow. This differential growth rate could be reflected in the transcriptome of these tissues as differential gene expression. Therefore, the effect of the dietary energy of milk replacer on the rumen microbiome and transcriptome is tissues related to growth should be examined.

5. Figures and Tables

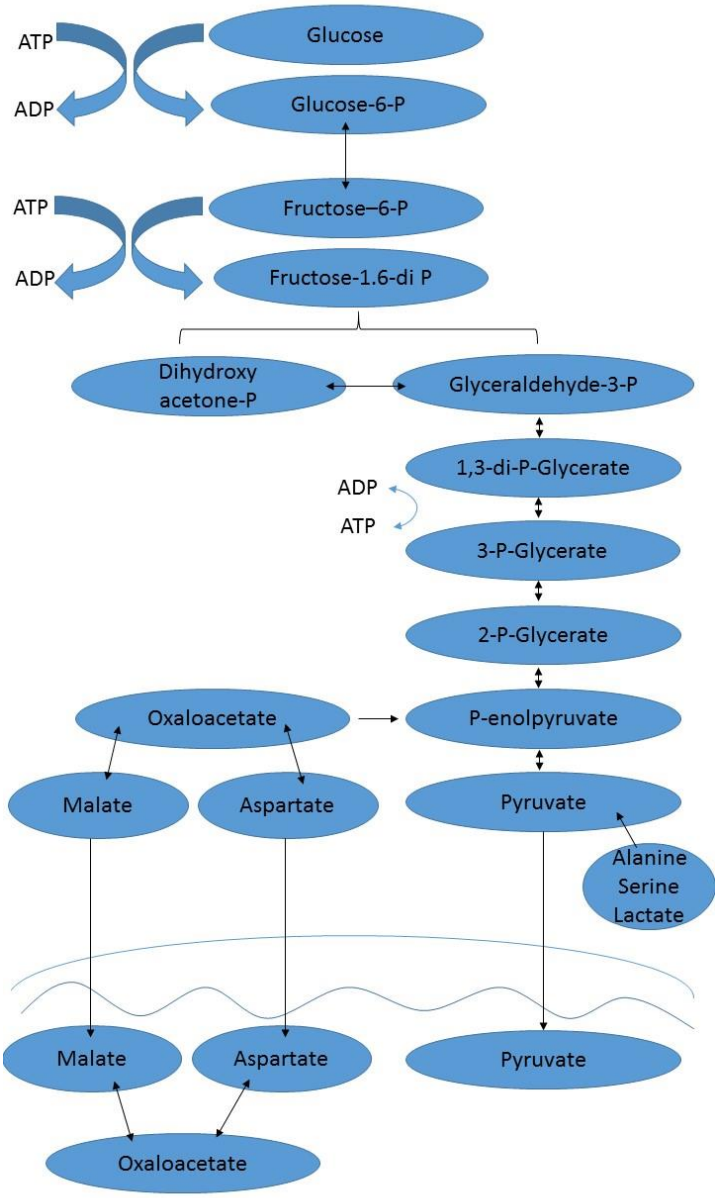

Figure 1.1

The molecular pathway of gluconeogenesis, generation of glucose within the cell (adapted from Hanson and Owen, 2004).

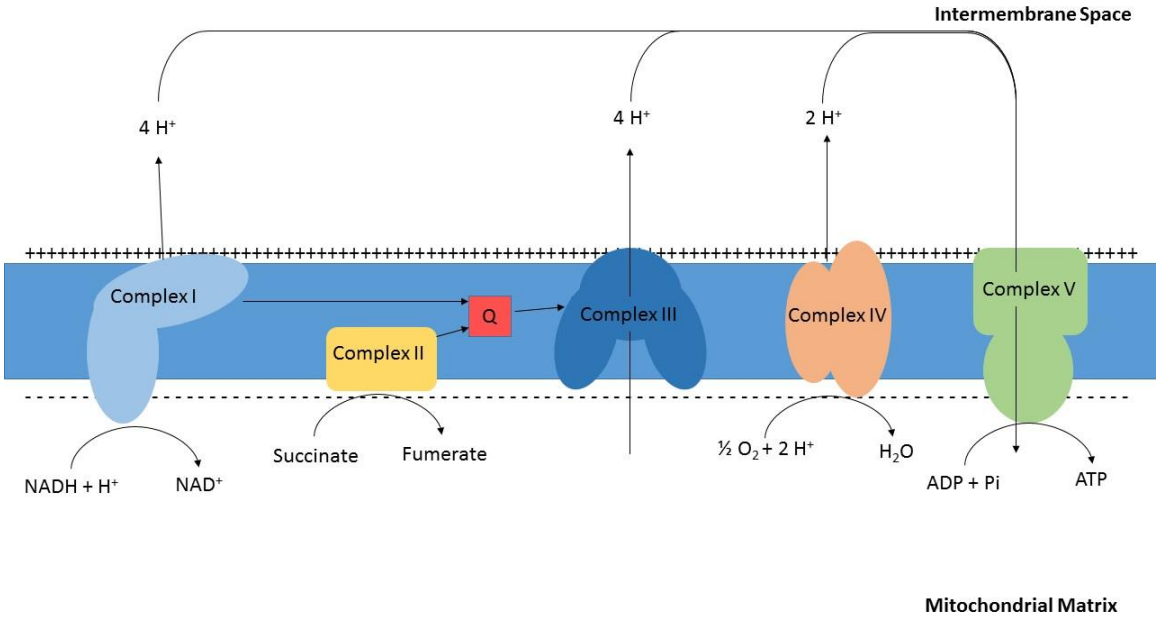

Figure 1.2: Molecular pathway for oxidative phosphorylation in the mitochondria of the cell. (adapted from Piombini et al., 2012)

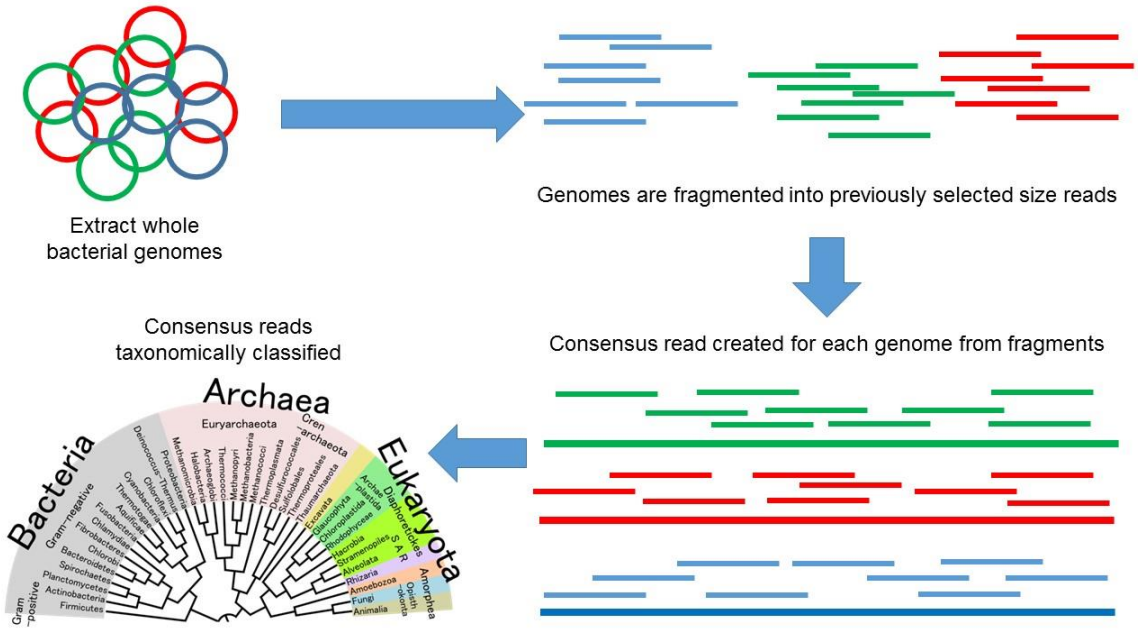

Figure 1.3: Overview of how whole genome sequencing is performed in a metagenomic analysis. Each color represents a different microbial organism present in the sample.

Table 1.1 Daily energy and protein requirements of young replacement calves fed only milk or milk replacer (NRC, 2001).

			Energy				Protein	
Live Weight (kg)	Gain (g)	Dry Matter Intake (kg)	NE_M (Mcal)	NE_G (Mcal)	ME (Mcal)	DE (Mcal)	ADP (g)	CP (g)
25	0	0.24	0.96	0	1.12	1.17	18	20
	200	0.32	0.96	0.26	1.50	1.56	65	70
	400	0.42	0.96	0.60	2.00	2.08	113	121
30	0	0.27	1.10	0	1.28	1.34	21	23
	200	0.36	1.10	0.28	1.69	1.76	68	73
	400	0.47	1.10	0.65	2.22	2.31	115	124
40	0	0.34	1.37	0	1.59	1.66	26	28
	200	0.43	1.37	0.31	2.04	2.13	73	79
	400	0.55	1.37	0.72	2.63	2.74	120	129
	600	0.69	1.37	1.16	3.28	3.41	168	180
45	0	0.37	1.49	0	1.74	1.81	28	30
	200	0.46	1.49	0.32	2.21	2.30	76	81
	400	0.59	1.49	0.75	2.82	2.94	123	132
	600	0.74	1.49	1.21	3.50	3.64	170	183
50	0	0.40	1.62	0	1.88	1.96	31	33
	200	0.45	1.62	0.34	2.37	2.47	78	84
	400	0.63	1.62	0.77	3.00	3.13	125	135
	600	0.78	1.62	1.26	3.70	3.86	173	185

Table 1.2 Comparing the cost of using whole milk or milk replacer when feeding pre-weaned calves (Jones and Heinrichs, 2017).

	Whole Milk	***Milk Replacer***
Crude protein (% dry matter)	25.5	20.7
Fat (% dry matter)	28.0	20.7
Cost per pound of dry matter	$1.30	$1.33
Cost per 22 kg of dry matter	$65	$66
Dry matter fed per calf (kg/d)	0.49	0.66
Crude protein fed per calf (kg/d), DM basis	0.12	0.14
Fat fed per calf (kg/d), DM basis	0.14	0.14
Cost per calf per day ($/calf/d)	$1.40	$1.58

Table 1.3: Milk replacer protein sources categorized based on their acceptability (BAMN, 2010). Acceptable sources are used in partial substitute of milk protein.

Preferred	Acceptable	Marginal
Whey protein concentrate	Soy protein isolate	Soy flour
Dried skim milk	Protein modified soy flour	Modified potato protein
Casein	Soy protein concentrate	
Dried whey	Animal plasma	
Dried whey product	Egg protein	
	Modified whey protein	

Table 1.4: Percentage of herds by primary factor used to determine when to wean heifers and herd size (USDA-APHIS, 2016).

	Small (30 - 99)		**Medium** (100 – 499)_		**Large** (500 or more)		**All operations**	
Primary Factor	Pct.	SE	Pct	SE	Pct	SE	Pct	SE
Consumed at least 0.90 kg of starter for 3 consecutive days	20.1	(2.0)	26.1	(2.5)	14.9	(1.9)	21.5	(1.5)
Reached the target weaning age	51.4	(2.6)	46.4	(2.8)	54.8	(2.8)	50.2	(1.8)
Reached the target weaning weight	21.6	(2.1)	21.3	(2.3)	19.9	(2.2)	21.3	(1.5)
Needed the space for other preweaned calves	3.0	(0.9)	5.2	(1.3)	6.6	(1.5)	4.0	(0.7)
Other	3.9	(1.0)	1.1	(0.5)	3.8	(1.1)	3.0	(0.7)

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CHAPTER 2

INCREASE IN DIETARY ENERGY OF PREWEANING DIET INCREASES RUMEN MICROBE DEVELOPMENT IN HOLSTEIN HEIFERS

1. **Abstract**

Proper management of a pre-weaned Holstein heifer is crucial to their development. Ensuring that a calf has the proper housing, environment, and nutrition is key ensuring the calf is physiologically developed enough when they reach maturity. The nutritional content of milk replacer has the greatest impact on a pre-weaned calf's development. As the calf grows, the microbes within their rumen develop and change with the ingestion of milk replacer and plant-based feeds. Children that consume lower levels of dietary energy tend to have a gut microbiome more similar to younger individuals. Therefore, we hypothesized that pre-weaned Holstein heifers consuming decreased dietary energy will have a different rumen microbial profile composition compared to heifers on an increased plane of nutrition. The objectives of this study were to 1) characterize rumen microbial profiles of calves fed differing levels of dietary energy, 2) determine if the rumen microbial profiles differed between diets, and 3) identify relationships between microbial phyla and growth. Holstein heifer calves (n = 12) were randomly assigned to 1 of 2 milk replacer diets: a restricted (R; 20.9% CP, 19.8% Fat; n = 6) or enhanced diet (E; 28.9% CP, 26.2% Fat; n = 6). Calves were euthanized and rumen fluid samples were collected at pre-weaning (8 wks; n = 6) or post-weaning (10 wks; n = 6) and stored at -80° C . Libraries were constructed from extracted DNA and DNASeq was conducted using a paired-end analysis at 100 bp using Illumina HiSeq 2500. Operational taxonomic unit (**OTU**) clustering analyses was conducted using the 16s rRNA Greengenes reference. A

PERMANOVA analysis was conducted in R to determine OTU populations for age and treatment. Microbiome composition differed ($P < 0.001$) between E and R calves, but there was no difference ($P = 0.761$) between pre-weaning and post-weaning calves. Bacteroidetes and Firmicutes represented the most abundant phyla for both E and R calves. Enhanced calves had 49.4% (5141 reads) Bacteriodetes and 36.4% (3789 reads) Firmicutes; whereas, R calves had 31.6% (2491 reads) Bacteriodetes and 41.1% (3236 reads) Firmicutes. Overall, varying levels of dietary energy in milk replacer affects rumen microbial composition in dairy heifers. The rumen ecology of R calves were similar to a 3 d old calf; whereas, the microbial composition of E calves was more similar to a mature calf.

2. **Introduction**

As the calf grows and transitions from pre-weaning to post-weaning, they need to be prepared to efficiently digest solid feeds from plant material. During the preweaning stage, the rumen, the portion of the stomach primarily responsible for digestion of cellulose, increases in size from 30% of the mass of the stomach at 2 wks to 80% of the mass of the stomach at 8 wks (Moran et al., 1993). While the exterior of the rumen changes in size, the more important change occurs inside the rumen. The rumen is not directly responsible for plant fiber digestion, but instead it relies on the assistance of microorganisms. These microorganisms change in composition from birth to weaning, with Proteobacteria being the dominant phyla at birth and Bacteroidetes being the dominant phyla at weaning and throughout mature life (Rey et al., 2013; Jami et al., 2013). While there has been evidence supporting some host control of the microbiome, the rumen microbiome can be changed by differences in the feed (Roehe et al., 2016;

Malmuthuge and Guan, 2017). Diets high in forages can cause more Bacteroidetes to be present, while diets high in concentrates tend to cause more Firmicutes to be present (Ellison et al., 2014).

The rumen microbiota represent one potential mechanism influencing a cow's feed efficiency status. Microbes can be introduced to the rumen via contact between the mother and newborn, vaginal births, exposure to feces, ingestion of milk, or direct transfer (Bryant and Small, 1958; Jayne-Williams, 1979; Fonty et al., 1987). Jami et al. (2014) found a strong correlation between the ratio of Firmicutes to Bacteriodetes and some phenotypic traits in the cow. They found a strong negative correlation between Bacteriodetes species and residual feed intake (**RFI**). This suggests a relationship between the rumen microbiome and feed efficiency of the cow. Through the rumen phylogenic profile of post-natal calves differs from the adult cow, bacterial organisms found in 1 wk old calves such as coliforms, lactobacilli, streptococci, and gram-negative faculatatively anearobic urease-positive rods survive in adult cattle (Bryant et al., 1958; Fonty et al., 1987). A small amount of milk will leak into the rumen during the first week of a calf's life, despite closure of the esophageal groove, providing an opportunity for microbial inoculation. Typical commercial milk replacers range from 20% to 24% CP and 18% to 24% fat (USDA-APHIS, 2016). Though energy levels provided in calf starter are based on NRC recommendations, calf growth and digestive tissue development is delayed on lower planes of nutrition (Geiger et al., 2016).

In humans, children who receive less energy or are malnourished have a gut microbiome more similar to a younger individual (Blanton et al., 2016). It is possible that calves that receive lower dietary energy could have a different rumen microbiome. This

change in the rumen microbiome could affect traits such RFI or milk-fat yield in the adult cows. Therefore, we hypothesized that increasing dietary energy of milk replacer in pre-weaned Holstein heifers will subsequently increase microbial abundance. Additionally, we hypothesize that microbial profiles will be correlated with calf and rumen growth. The objectives of this study were to 1) characterize rumen microbial profiles of calves fed differing levels of dietary energy, 2) determine if the rumen microbial profiles differed between diets, and 3) identify relationships between microbial phyla and growth.

3 **Materials and Methods**

3.1 *Study Design and Sample Source*

Samples used in this study were obtained from calves originally enrolled in IACUC protocol #14-045-DASC at Virginia Tech. Full details of the calf experiment are published elsewhere (Geiger et al., 2016a, 2016b). Briefly, 36 Holstein heifer calves (6.0 ± 2 d old and 39.03 ± 4.43 kg BW) were reared on one of two treatment diets (n = 18/treatment). Dietary treatments were either a restricted milk replacer diet fed at 0.44 kg powder dry matter (DM)/day [**R**; 20.9% crude protein (CP), 19.8% fat, DM basis], or an enhanced MR fed at 1.08 kg powder DM/d (**E**; 28.9% CP, 26.2% fat, DM basis). MR was fed at 15% solids in 2 equal portions, twice daily at 0600 and 1700 h for the first 7 wk of trial. At wk 8, heifers were fed half the usual amount 1× daily at 1700 h to prepare for weaning. Calves were completely weaned at the end of wk 8.

A common calf starter (25.6% CP, 4.0% fat, 19.8% NDF, DM basis) was offered to calves on both treatments after wk 4, in a controlled manner. The R-fed calves were offered starter in the amount of what was consumed by E-fed calves on the previous day.

Calves were individually fed and housed in non-bedded outdoor hutches for the duration of the trial. Daily MR and starter intakes were recorded. Water was available at all times and water intake was not recorded. Growth measurements (BW, hip height, withers height) were obtained weekly; ADG was calculated weekly.

At the end of week 8, 12 calves (E = 6, R = 6), were euthanized for sample collection, while the remaining 24 calves (E = 12, R = 12) were placed on ad libitum access to starter for an additional 2 wk. At the end of week 10, remaining calves were euthanized for sample collection. One calf died within 48 h of arrival and was not replaced.

3.2 *Rumen sample collection*

All calves received their last feeding the evening prior to their scheduled harvest (~16 h pre-harvest). At time of harvest, heifers were euthanized using a commercial phenobarbital solution administered intravenously (Fatal-Plus, 10 mg/kg BW, Vortech Pharmaceuticals, Dearborn, MI), exsanguinated, and subjected to organ collection. Prior to organ collection, exsanguinated BW (carcass weight) was determined. Full and empty rumen weights as well as rumen pH were measured at time of harvesting. Approximately 6.0 ml of mixed rumen contents were collected from each calf and snap frozen using liquid nitrogen in individual cryotubes (2.0 mL/tube). Tubes were stored at -80 °C for longterm storage.

3.3 *DNA extraction and sequencing*

Regardless of harvest age (8 wk or 10 wk), enhanced calves with the highest lifetime ADG and G:F (n = 6) and R calves with the lowest lifetime ADG and G:F (n = 6)

were selected for metagenomic analyses. The QIAGEN DNA Stool Kit (QIAGEN, Germantown, MD) was used to extract DNA from rumen content samples. Concentrations were increased by re-precipitating extracted DNA. Quantity and quality of DNA were examined using the Agilent DNA 1000 kit (Agilent Technologies, Santa Clara, CA) before being sequenced. Samples were then sent to University of Missouri DNA Core lab overnight on dry ice for library construction and sequencing. Whole metagenome sequencing was conducted using Illumina Hiseq 2500 (Illumina, San Diego, CA), 2 x 100 bp paired-end reads.

3.4 *Statistical analysis*

Bacterial 16s rRNA genes were extracted from sequencing files and went through operational taxonomic unit (**OTU**) clustering analysis using Kraken (Johns Hopkins Center for Computational Biology, Baltimore, MD). Reads were aligned and assigned taxonomies using 97%-similarity to the Greengenes (Greengenes, Berkeley, CA) reference genome. A Permutational Analysis of Variance (PERMANOVA) was performed between treatment groups in R using logLIK (R, Pinheiro and Bates) to examine if there was difference in microbial content of the rumen samples. The Pearson CORR procedure in SAS was performed to determine phenotypic correlations between the calf phenotypes (e.g., ADG, G:F, full and empty rumen weight, and rumen pH) and phyla abundance .

4. **Results**

4.1 *Calf growth and performance*

Calves fed the R diet consumed more starter DM during the preweaning period than E calves (286 g/d and 237 g/d, respectively; $P < 0.01$). However, overall total weekly

dry matter intake (**DMI**) was greater for E calves than R calves during the preweaning period (8.80 kg and 5.08 kg, respectively; $P < 0.01$). Calves fed the E diet consumed more milk replacer DM, CP, fat, and energy than R calves. Calves fed the R diet consumed more starter DM during the postweaning period than E calves (1301 g/d and 1257 g/d, respectively; $P < 0.01$).

Initial BW did not differ between the two treatments. After the diet had been administered for 2 wks, E calves were heavier than R calves and remained so for the rest of the experiment. After the diet had been administered for 1 wk, ADG was greater in E calves than R calves through wk 7 (1.00 kg/d and 0.41 kg/d, respectively; $P < 0.01$). There was no difference in ADG during wk 8 or during the postweaning period.

4.2 *Microbial Composition*

A total of 1807 unique OTUs were identified between diets. Enhanced calves had a total of 74,909 reads sequenced, while R calves had a total of 62,491 reads sequenced. Of the total amount of OTUs, only 440 (36,333 reads, 24.3%) were aligned to a taxonomy other than "unclassified bacterium." There was a difference ($P < 0.001$) in the overall rumen microbial population between diets (Figure 2.1). Two phyla, Bacteriodetes and Firmicutes, dominated the rumen microbiome and were affected by treatment. Bacteriodetes was the dominant phylum within E calves (E = 5141, 49.423%; R = 2491, 31.644%), while Firmicutes was the dominant phylum in R calves (E = 3789, 36.426%; R = 3236, 41.108) (Table 2.1). Bacteriodetes is the dominant phylum within E calves, while Firmicutes is the dominant phylum in R calves. There was an increase in the amount of Proteobacteria and Spirochetes in R calves when compared to E calves (Table 2.1). Individual variation in rumen microbiome was present (Figure 2.2, Figure 2.3).

4.3 *Phenotypic Correlation*

There was a strong, negative phenotypic correlation between Proteobacteria and both full (r = -0.79, P = 0.003) and empty rumen weights (r = -0.70, P = 0.017). There was also a strong negative phenotypic correlation between Spirochetes and ADG (r = -0.610, P = 0.035; Figure 2.4). Within the R calves, there was a strong negative correlation between the full rumen weight and both Bacteriodetes (r = -0.87, P = 0.028) and Proteobacteria (r = -0.87, P = 0.023). Within E calves, there was a strong negative correlation between Spirochetes and empty rumen weight (r = -0.97, P = 0.007) and Proteobacteria and both full rumen weight (r = -0.88, P = 0.048) and empty rumen weight (r = -0.85, P = 0.067). There was a strong positive correlation between Firmicutes and ADG (r = 0.91, P = 0.013).

5 **Discussion**

Initially, it was thought that the calf rumen was sterile at the time of birth, with inoculation occurring primarily through ingestion of bacteria, protozoa, fungi, and archaea (Ziolecki and Briggs, 1961; Stewart et al., 1988). Newer data indicate anaerobic rumen microorganisms are present in the calf rumen at just 1-2 d of age (Fonty et al., 1987; Morvan et al., 1994; Anderson et al., 1987). While the majority of rumen bacteria are present at 14 d, a portion of those essential for rumen function are present at just 1 d of age (Li et al., 2012; Jami et al., 2013). The bacteria present from 1 – 3 d are mostly Proteobacteria, specifically *Succinivibrio*, which are primarily responsible for plant digestion and carbon dioxide fixation (O' Herrin et al., 1993). However, it has been difficult to identify the function of some Proteobacteria because not every species has been cultured outside of the rumen (Creevey et al., 2014). As the calf matures, the

majority of bacteria shift from Proteobacteria to Bacteriodetes and Firmicutes (Rey et al., 2013). These are responsible for fermentation and protein breakdown (Wallace et al., 1997; Ricke and Schaefer, 1996). By 7 wks of age, Bacteriodetes become the major phylum in the rumen (Rey et al., 2013; Jami et al., 2013). Our research confirms previous research that the primary microbes present in the rumen for E and R calves 8 to 10 wks of age were Bacteriodetes and Firmicutes.

Interestingly, we observed 17% more Proteobacteria in R calves. We expected low levels of Proteobacteria in the calf rumen by 8 to 10 wks of age, but the high percentage of Proteobacteria in R calves may signify that these calves had a less developed rumen. To our knowledge, this is the first study examining the effects of dietary energy on rumen microbiome development. Research in humans found that 2 to 3 yr old children that were malnourished tended to have decreased total OTU in the gut microbiome than in healthy individuals (Monira et al., 2011). For the malnourished children, the Proteobacteria and Bacteriodetes comprised 46% and 18% of the microbiome of fecal samples, respectively; whereas, in healthy children Proteobacteria and Bacteriodetes comprised 5% and 44%, respectively. Subramanian et al. (2014) also found that malnourished children had an immature gut microbiome when compared to healthy children. While the cow's rumen functions differently than a human's stomach, the fecal samples from human children are more representative of their large intestine. Both the rumen and large intestine function as the main sites of fermentation, so it is possible that the human fecal microbiome could be representative of what occurs in the rumen and vice versa (Macfarlane and Macfarlane, 2011). Based on NRC requirements, R calves were not malnourished, but the increase in Proteobacteria in R calves combined

with the reduced growth rates (Geiger et al., 2016) reflects a trend similar to malnourishment. We suggest that the microbial composition of the rumen of the R calves is more similar to a 3 d old calf, indicating that dietary energy affects development of the rumen bacteria and possibly the rumen.

While the rumen microbiome is partially established after the first few days of life, diet has the greatest effect on the calf rumen microbiome between 9 d and 15 d (Li et al., 2012; Jami et al., 2013; Rey et al., 2013). Our calves were placed on their respective diets at 7 d, so the continued ingestion of the E or R diet from 9 d to 15 d and beyond could be one source of microbiome differentiation. However, if the calves were removed from their respective milk replacer diets and given the same starter diet at approximately the same age, the microbiome should be more similar. But this may not be the case in calves where the diet is changed early in life. While the early stages of a calf's life are critical for the establishment of the rumen microbiota, this does not mean there is no ability to change the microbiota. Producers can use probiotics in heifers and cows to manipulate the rumen microbial composition (Ellison et al., 2013; Uyeno et al., 2015). However, this solution is not permanent. Once the probiotic is removed from the diet, the microbiome shifts back to the original composition as soon as the next week (Uyeno et al., 2015). However, there may be a specific window of time in which permanent change to the rumen can occur and this has yet to be classified. Further research must be be conducted to examine the long-term effects of dietary energy on the rumen microbiome. There is a possibility that the R calves would never establish a rumen microbiome similar to E calves due to microbial programming and fixation during the first weeks of life.

Proteobacteria contains a large group of *Enterobacteria*; therefore, increases observed in R calves could possibly elicit an immune response. In a fully developed rumen, there are a series of protective mechanisms that allow microorganisms to coexist in the rumen (Hooper et al., 2012). However, these mechanisms need to be learned or trained in early stages of life. While mechanisms involved in accepting the first microbial colonizers in the rumen is unknown, colostrum does act as a method of "teaching" the calf. Levels of antibodies against *Butyrivibrio*, *Streptococci*, *Lactobacilli* are similar in the dam colostrum and serum, which were subsequently observed in the calf's serum (Sharpe et al., 1977). If the colostrum can partially affect or teach the calf protective mechanisms, it might be possible to incorporate a similar approach to stimulate microbial growth in calves. The amount of CP and fat in the milk replacer could be one method of stimulating these mechanisms. The R diet might not have allowed for these unknown mechanisms to fully develop by the time of tissue collection, thus allowing for Proteobacteria to grow more than in the E calves.

One mechanism that does change during weaning of the calves is expression of toll-like receptors (*TLR*), *β-defensin*, and *peptidoglycan recognition protein 1* (*PGLYRP1*). At weaning, *TLR* in the rumen are down-regulated, while *β-defensin* and *PGLYRP1* expression is increased (Malmuthuge et al., 2012). Though not well characterized, it is hypothesized that *TLR* are important for immune response in young calf rumens. But as the calf ages, other innate immune responses become more prominent. It could be that once the microbial composition of the calf reaches a certain point, *TLR* expression decreases, allowing for further microbial development. The E calves' microbial composition could have reached this point earlier than the R calves and

allowed for a more mature microbial composition. Unfortunately, expression of *TLR* with varying microbiome compositions has yet to be studied.

By weaning, calves must have a mature rumen microbial population prepared to breakdown solid foods. Based on what is present in older cows, the bacteria responsible for rumen digestion seem to be in either the Firmicutes or Bacteriodetes phyla (Rey et al., 2013; Jami et al., 2013). From this study, it appears that an increase in dietary energy of the milk replacer promotes the development and maturation of microbial populations, which is reflective of the adult cow rumen microecology. The increase of phyla like Proteobacteria and Spirochetes in R calves makes their rumen microbial profile more similar to a 3 d old calf than a 8 to 10 wk old calf (Rey et al., 2013). While the diet of the R calves does have less energy and protein available than the E calf diet, it still falls within NRC requirements. Additionally, the smaller rumen size in R calves is also more similar to younger calves. The negative phenotypic correlation between smaller rumen size and Proteobacteria and Spirochetes indicates that the maturity of the microbiome affects the development of the rumen. Producers in the United States use a milk replacer composition similar to R calves, with about 51% percent of large herd (> 500 cows) using a similar milk replacer in 2014 (USDA-APHIS, 2016). It might be possible that producers are starting their calves out with a detriment to their digestive ability and negatively impacting the herd early on. While it is possible the rumen microbial population could mature to the proper level after weaning, the period of time it would take for that microbial population to mature could account for a significant amount of money unnecessarily spent for feed.

6 **Conclusions**

Increases in dietary energy of milk replacer changes the rumen microbial composition of pre-weaned Holstein heifers. In E calves, the dominant phylum present was Bacteroides, which is consistent with calves at that age or older. In R calves, the dominant phylum was Firmicutes, but there was also an increase in Proteobacteria and Spirochetes. Restricted calves also had smaller rumen weights, which was correlated with increased levels of Proteobacteria. The rumen microbial and phenotypic profile of R calves is consistent with calves around 3 d of age. While the R diet does fall within NRC requirements, we suggest that requirements be revised to reflect current management procedures and to ensure proper gut health of dairy calves.

7. Figures and Tables

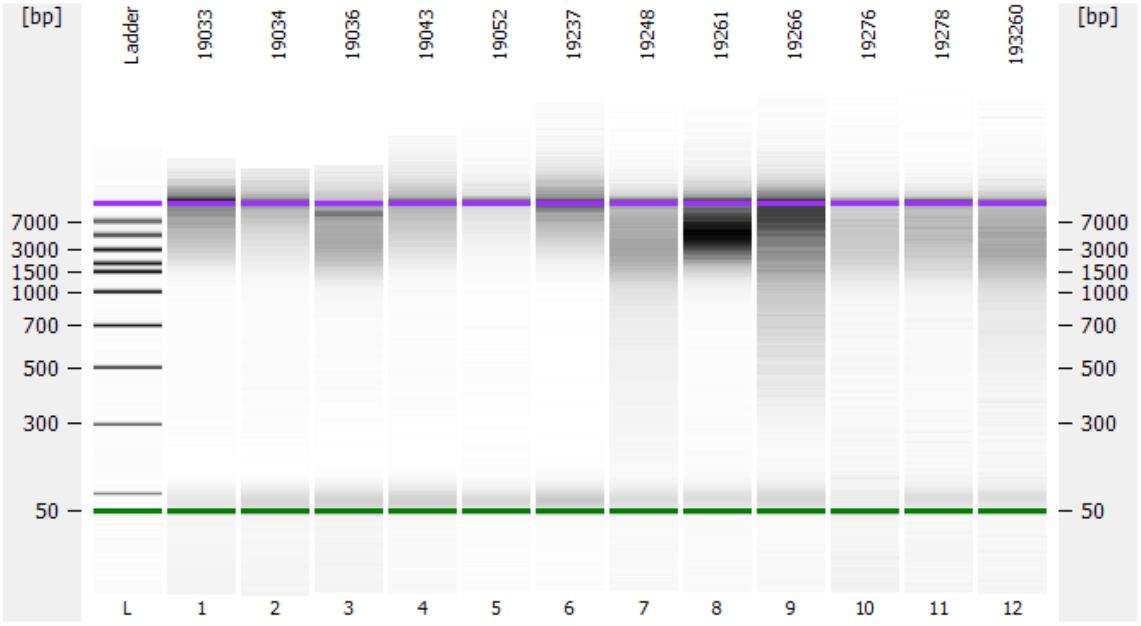
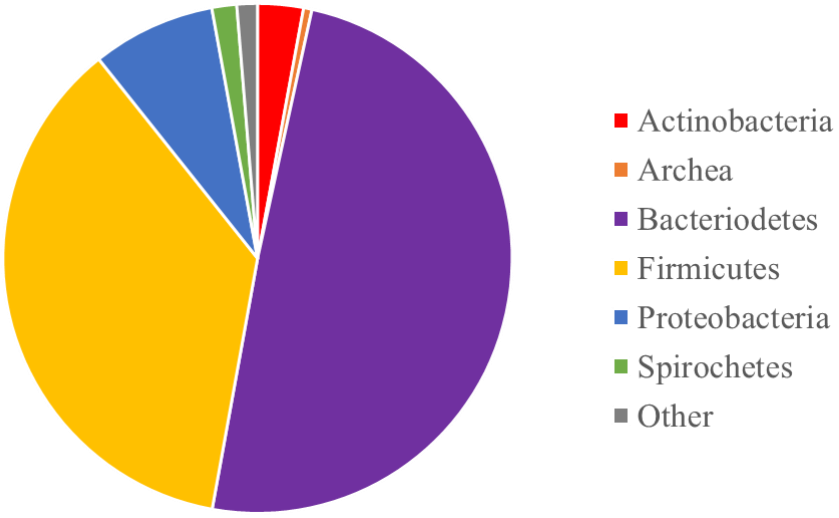


Figure 2.1 Gel image of the extracted rumen DNA examining the samples for quality. Each column represents a sample. If the DNA is of acceptable quality, the majority of the banding should fall along the purple bar within each column. Sample 19261 is the only sample that has banding below the bar, but this was still deemed acceptable for sequencing.

A)



B)

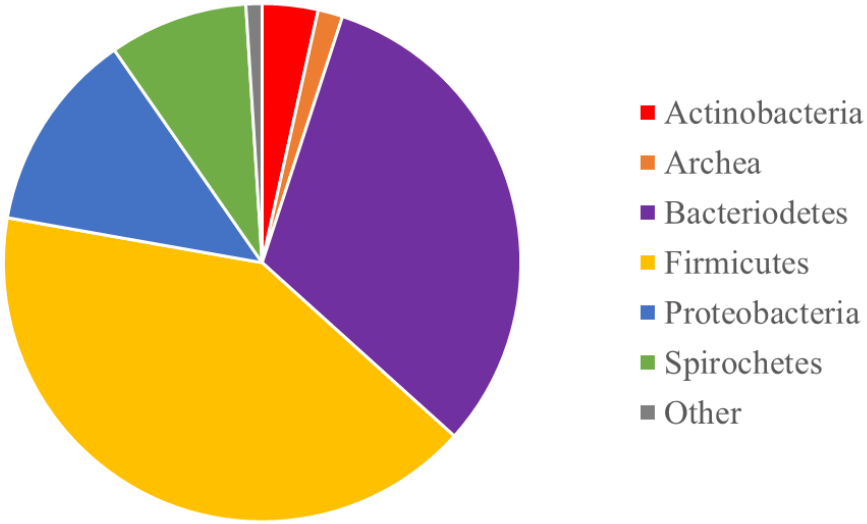


Figure 2.2 Operational taxonomic unit (OTU) clustering for calves fed an Enhanced (E; n = 6) or Restricted (R; n = 6) diet. **A)** Rumen microbial composition of E calves. **B)** Rumen microbial composition of R calves. There was an increase in abundance of

Bacteriodetes in E calves compared to R calves; whereas, there were more Firmicutes present in R calves compared to E calves.

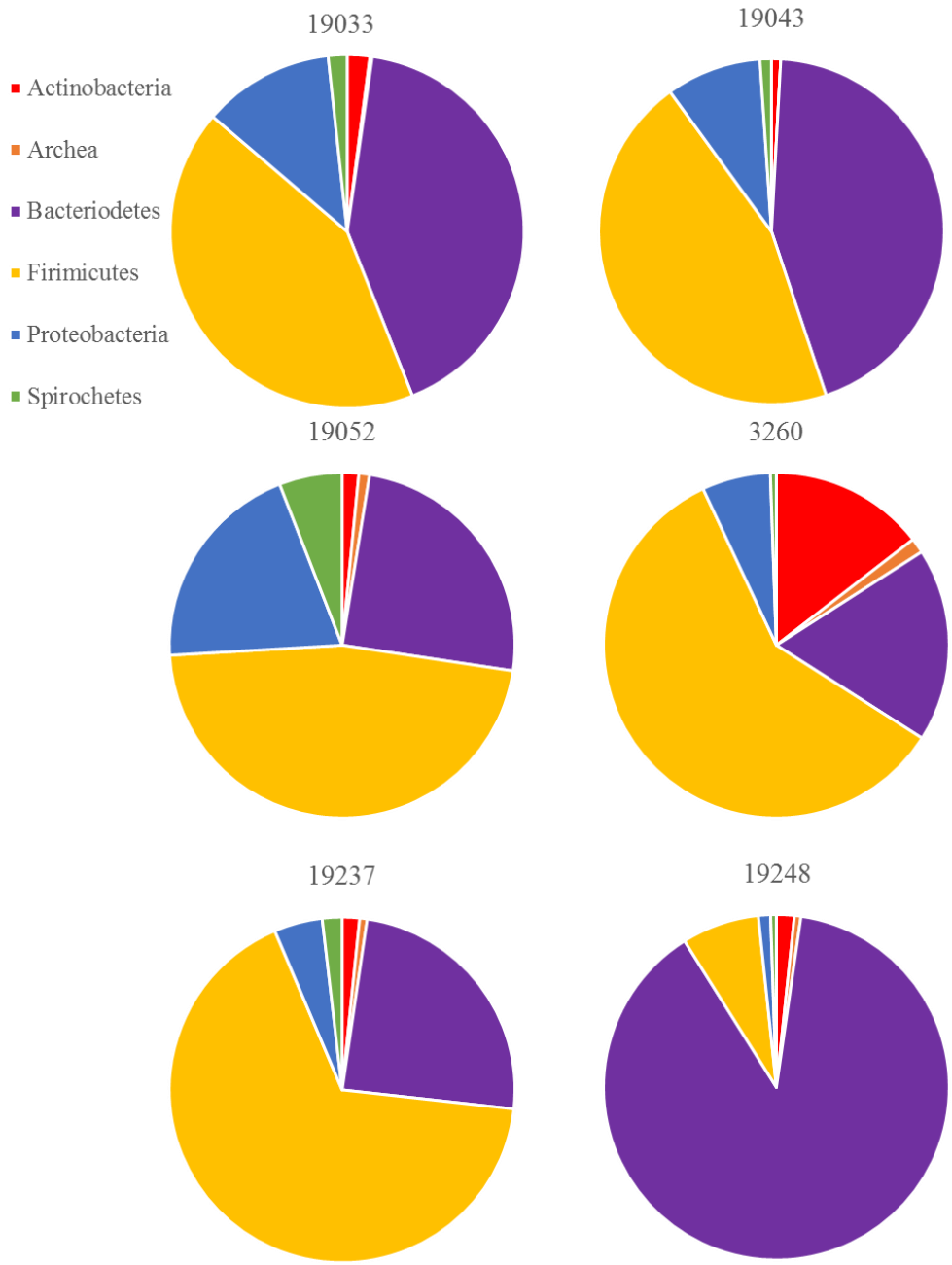


Figure 2.3 Operational taxonomic unit (OTU) clustering for calves fed an Enhanced (E; n = 6) diet. Calves 19033, 19043, 19052, and 3260 had samples collected at 8 wks (weaning). Calves 19237 and 19248 had samples collected at 10 wks (post-weaning).

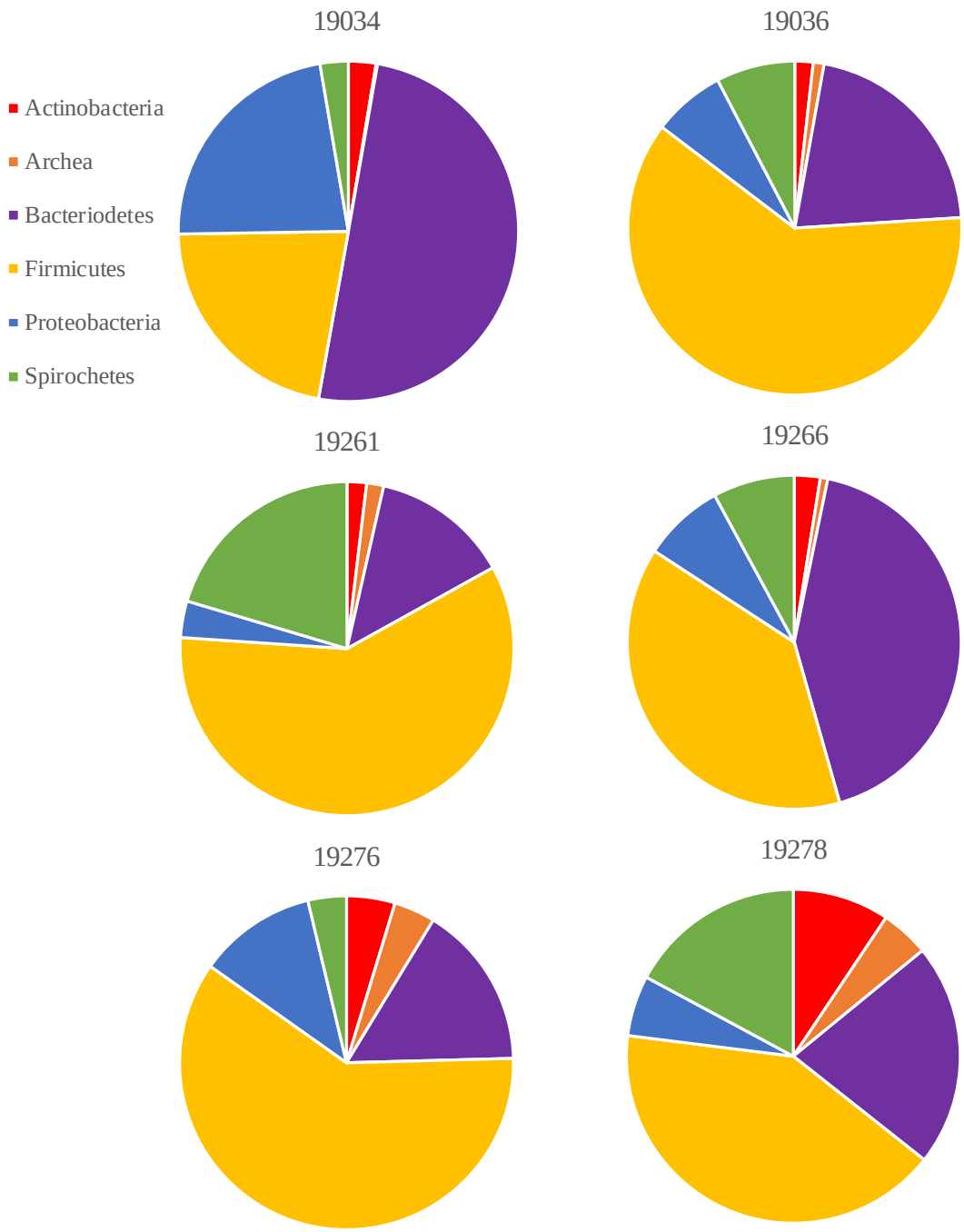


Figure 2.4 Operational taxonomic unit (OTU) clustering for calves fed a Restricted (E; n = 6) diet. Calves 19034 and 19036 had samples collected at 8 wks (weaning). Calves 19261, 19266, 19276, and 19278 had samples collected at 10 wks (post-weaning).

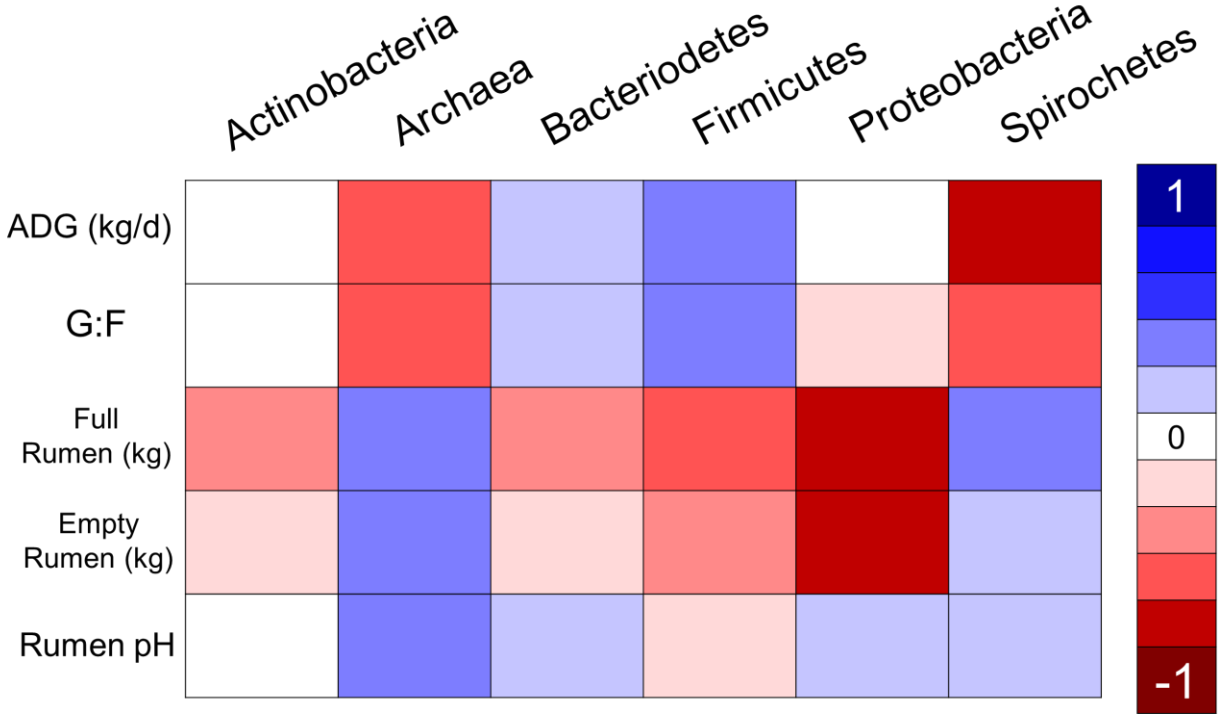


Figure 2.5 Pearson correlations between the operational taxonomic unit (OTU) clustering counts and calf measures for ADG, G:F, full and empty rumen weight, and rumen pH.

Table 2.1. Operational taxonomic unit (OTU) percentages and abundance for the major phyla of bacteria from rumen fluid of calves fed an Enhanced (E) or Restricted (R) diet[1].

	Enhanced (n = 6)		**Restricted** (n = 6)	
Phylum	**Percentage**	**OTU Abundance**	**Percentage**	**OTU Abundance**
Actinobacteria	2.903	302	3.480	274
Archea	0.529	55	1.550	122
Bacteriodetes	49.423	5141	31.644	2491
Firmicutes	36.426	3789	41.108	3236
Proteobacteria	7.835	815	12.538	987
Spirochetes	1.577	164	8.676	683
Other[2]	1.307	134	1.004	78

1 Enhanced diet (E): 28% CP, 25% Fat. Restricted Diet (R): 20% CP, 20% Fat. Percentages within diet were calculated based on the total number of reads present within the OTUs assigned taxonomies.

2 Other refers to phyla with only one OTU present: Cyanobacteria, Chloroflexi, Deferribacteres, Deinococcus-Thermus, Lentisphaerae, Tenericutes, and Verrumicrobiales.

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CHAPTER 3

CHANGE IN DIETARY ENERGY DURING PRE-WEANING PERIOD CHANGES TRASCRIPT PROFILE IN TISSUES RELATED TO GROWTH IN HOLSTEIN HEIFERS

1 **Abstract**

We hypothesized that feeding milk replacer varying in dietary energy content would elicit differential expression of genes within pathways associated with growth and metabolism. The objectives of this study were to 1) identify transcripts differentially expressed in tissues related to growth and metabolism in pre-weaned dairy heifers, and 2) determine the growth and metabolic pathways influenced by these transcripts. Pre-weaned Holstein heifers (n = 12; age 6 d ± 0.02) were randomly assigned to 1 of 2 milk replacer diets: Enhanced (E; 28.9% CP, 26.2% Fat; n = 6), or Restricted (R; 20.9% CP, 19.8% Fat; n = 6). After 8 wks, samples from longissimus dorsi (LD), adipose (A), and liver (L) tissues were collected, snap frozen and stored at -80° C. Libraries were constructed from extracted RNA for RNA-Seq analyses. Average daily gain (ADG) and gain-to-feed ratio (G:F) were calculated for each calf. Analysis of ADG and G:F was performed using PROC GLM in SAS with diet as the main effect; E calves had increased ADG and G:F. RNA-Seq analysis was performed using CLC Genomics Workbench and the Robinson and Smith Exact Test was used to identify differentially expressed genes between diets. There were 238 differentially expressed genes in A, 227 in LD, and 40 in L. Of the differentially expressed genes, 10 appeared in at least 2 tissues. PANTHER was used to identify functional categories of differentially expressed genes. The majority of genes were associated with metabolic processes (A = 112, 26.7%; L = 16, 32.0%; LD = 81, 34.0%) or cellular processes (A = 93, 22.1%; L = 13, 26.0%; LD = 73, 30.7%). In E calves, upregulated genes included those regulating NADH dehydrogenation (LD = 17, A

= 5; i.e. *ND1*, *ND4*), gluconeogenesis (LD = 2, A = 6; i.e. *ALDOB*, *PCK2*), and cell proliferation (LD = 2, A = 3; i.e. *GADD45A, CDKN1A*). This change in regulation of cell cycle and ATP synthesis in response to dietary energy could explain the difference in ADG between diets.

2. **Introduction**

The majority of milk replacers contain 20% crude protein (CP) and 20% fat, and are fed at 15% body weight (BW) of the calf (USDA-APHIS, 2008). While this is within NRC energy requirements, there is currently a shift towards feeding increased levels of both CP and fat (USDA-APHIS, 2010; USDA-APHIS, 2016). Changes in CP and fat in the milk replacer impact tissue growth and development; however, the underlying mechanisms at the cellular level are unknown.

The transcriptome of the calf is the collection of mRNA transcripts expressed in the animal at a specific point (Adams, 2008). This transcriptome can vary based on age of the calf, size of the calf, the environment they are being raised in, and their physiological state (Canovas et al., 2014; Guo et al., 2015). Because the transcriptome is different in each type of tissue, there are multiple transcriptomes within one animal (Adams, 2008). Because the transcriptome of the calf can have so much variability based on external factors, studies focus on how these factors affect gene expression in the calf. Guo et al. (2015) identified possible markers to link to muscle growth rate in cattle. They found a correlation between average daily gain (ADG)/kg and 66 cell cycle process genes as well as 46 extracellular matrix organization genes. Akbar et al. (2013) found a difference in expression of oxidative phosphorylation genes in the liver of cows under feed restriction.

An increase in CP and fat content in milk replacer can improve the ADG in calves. It is possible that calves fed diets with differing levels of CP and fat could have differentially expressed genes in muscle, adipose and liver. We hypothesized that genes within key growth and metabolic pathways will be differentially expressed between calves fed an energy restricted diet compared to calves fed an energy enhanced diet. The objectives of this study were to 1) identify transcripts differentially expressed in tissues related to growth and metabolism in pre-weaned dairy heifers, and 2) determine the growth and metabolic pathways influenced by these transcripts.

3. **Materials and Methods**

3.1 *Study design and sample source*

Samples used in this study were obtained from calves originally enrolled in IACUC protocol #14-045-DASC at Virginia Tech. Full details of the calf experiment are published elsewhere (Geiger et al., 2016a, 2016b). Briefly, 12 Holstein heifer calves (6.0 ± 2 d old and 39.03 ± 4.43 kg BW) were reared on one of two treatment diets (n = 6/treatment). Dietary treatments were either a restricted milk replacer diet fed at 0.44 kg powder dry matter (DM)/day [**R**; 20.9% crude protein (CP), 19.8% fat, DM basis], or an enhanced MR fed at 1.08 kg powder DM/d (**E**; 28.9% CP, 26.2% fat, DM basis). MR was fed at 15% solids in 2 equal portions, twice daily at 0600 and 1700 h for the first 7 wk of trial. At wk 8, heifers were fed half the usual amount 1× daily at 1700 h to prepare for weaning. Calves were completely weaned at the end of wk 8.

A common calf starter (25.6% CP, 4.0% fat, 19.8% NDF, DM basis) was offered to calves on both treatments after wk 4, in a controlled manner. The R-fed calves were offered starter in the amount of what was consumed by E-fed calves on the previous day.

Calves were individually fed and housed in non-bedded outdoor hutches for the duration of the trial. Daily milk replacer and starter intakes were recorded. Water was available at all times and water intake was not recorded. Growth measurements (BW, hip height, withers height) were obtained weekly; ADG was calculated weekly.

At the end of week 8, the 12 calves (E = 6, R = 6), were euthanized for sample collection. Calves were fasted 12 h prior to the time of tissue harvest. The calves were euthanized and tissues were harvested at weaning. Calves were euthanized at Virginia Tech's Veterinary Facility (approximately 1 mi from their housing). Calves were harvested using a commercial phenobarbital solution administered intravenously (Fatal-Plus, 10 mg/kg BW, Vortech Pharmaceuticals, Dearborn, MI), exsanguinated, and subjected to organ collection. When a calf was euthanized, tissue samples from the liver, adipose tissues from the kidney, and longissimus dorsi (LD) were collected and tissue weights were collected for liver and kidneys. Samples were snap frozen in liquid nitrogen and stored at -80 ºC until further processing.

3.2 *Statistical Analysis*

A PROC GLM was performed in SAS (SAS 193 Institute, INC., Cary, NC) to analyze ADG and G:F between diets. Main effect was diet. Carcass weight, liver weight, and kidney weight were already analyzed in Geiger et al. (2016) using a PROC GLIMMIX in SAS (SAS 193 Institute, INC., Cary, NC). Main effects were diet, time (week or day), harvest date (where appropriate), and their interactions. Significance was declared when $P < 0.05$.

3.3 *RNA Extraction and Sequencing*

RNA was extracted from each sample using a TRI Reagent Protocol (Sigma-Aldrich; St. Louis, MO) and then cleaned to remove any remaining contaminants using the QIAGEN RNeasy Mini Kit (Qiagen; Germantown, MD). The samples were analyzed for quality and concentration using both the Thermo Scientific Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA) and the Agilent RNA 6000 Nano kit with a 2100 Agilent Bio analyzer (Agilent Technologies; Santa Clara, CA). Samples had a RNA integrity number (RIN) value of 7 before being sequenced (Table 3.1). Samples were sent to University of Missouri's DNA Core Facility to undergo library construction and sequencing. The samples were sequenced using an Illumina NextSeq 500 (Illumina; San Diego, CA) at 1x75 single-end reads. Sequence analyses generated ~25 million reads per sample over 3 runs.

3.4 *RNA-seq Analysis*

Sequence reads were analyzed using CLC Genomics Workbench (Qiagen; Germantown, MD). Reads were imported in CLC genomics workbench and the 2 adapters were trimmed from the sequences. Sequences were then aligned to the UMD_3.1 *Bos taurus* reference genome and annotation from Ensembl. An empirical analysis of differential gene expression was performed using the Robinson and Smyth Exact Test (Robinson and Smyth, 2007). A negative binomial distribution (NB) was assumed. When *Yij* was the observed counts for the diet *i* and library *j* for a sequence,

$$Yij \sim \mathrm{NB}(\mu ij, \Phi)$$

where Φ is the dispersion and $\mu ij = mij\ \lambda i$, with mij being the library size for sample *j* and λi the true relative abundance of the RNA sequence in diet *i* (Nettleton and Datta,

2014). Volcano plots were created for each tissue using the log-transformed fold change (FC) and log-transformed p-value. Transcripts with a FDR corrected *P*-value ≤ 0.05 and RPKM ≥ 2 between diets were considered significantly differentially expressed.

3.5 *PANTHER and KEGG Pathway Analysis*

Differentially expressed transcripts from each tissue were analyzed using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) database to examine the biological functions associated with each gene. This also served as a functional annotation for each gene. The same transcripts underwent a pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) to analyze the biological pathways most commonly associated with the differentially expressed genes. The list of differentially expressed genes for each tissue was entered into the KEGG Pathway function. Pathways with less than 3 differentially expressed genes involved in the pathway were ignored. Pathways of interest were those that contained the highest number of differentially expressed genes.

4. **Results**

4.1 *Calf Growth and Performance*

Calves fed the R diet consumed more starter DM during the preweaning period than E calves (286 g/d and 237 g/d, respectively; $P < 0.01$). However, overall total weekly dry matter intake (**DMI**) was greater for E calves than R calves during the preweaning period (8.80 kg and 5.08 kg, respectively; $P < 0.01$). Calves fed the E diet consumed more milk replacer DM, CP, fat, and energy than R calves. Calves fed the R diet consumed more starter DM during the postweaning period than E calves (1301 g/d and 1257 g/d, respectively; $P < 0.01$).

Initial BW did not differ between the two treatments. After the diet had been administered for 2 wks, E calves were heavier than R calves and remained so for the rest of the experiment. At the time of tissue collection, carcass weights, liver weights, and kidney weights were measured. Additionally, ADG and G:F ratios were measured weekly (Table 3.2). Average daily gain and G:F was increased ($P \leq 0.007$) by 29% and 60% in E calves compared to R calves, respectively. Additionally, we observed increases ($P < 0.05$) in carcass weight, liver weight, liver weight per g/kg of BW, and average kidney weight in E calves compared to R calves.

4.2 *Differential gene expression*

The Robinson and Smith Exact Test was used to measure differential gene expression. Additionally, volcano plots were created to visualize differential expression in each tissue (Figure 3.1, 3.2, 3.3). We observed that of the differentially expressed genes between all the tissues in E and R calves, 238 genes were found in adipose tissue, 227 genes were found in LD tissue, and 40 genes were found in liver tissue. In adipose, the 5 genes with the greatest fold change (FC) were transglutaminase-3 (*TC3*, FC = 173.39, $P = 0.003$), a gene associated with lipid binding *(ENSBTAG00000009144*, FC = 119.50, $P = 0.003$), tetratricopeptide repeat domain 25 (*TTC25*, FC = 58.13, $P = 0.008$), keratin 79 (*KRT79*, FC = 55.61, $P < 0.001$), and Kelch domain containing 7A (*KLHDC7A*, FC = 55.31, $P < 0.001$). These genes were all upregulated in R calves. In LD, the 5 genes with the greatest FC were hypoxia induced factor 3 alpha subunit (*HIF3A*, FC = 82.89, $P < 0.001$), R-Spondin 3 (*RSPO3*, FC = 57.23, $P = 0.030$), metallothionein 3 (*MT3*, FC = 56.25, $P <0.001$), WD repeat domain 86 (*WDR86*, FC = 47.21, $P = 0.032$), and a gene that negatively regulated peptidease activity

(*ENSBTAG0000002525*, FC = 46.58, *P* = 0.011). These genes were all upregulated in R calves. In liver, the 5 genes with the greatest FC were oligoadenylate synthetase 1 (*OAS1X*, FC = -91.71, *P* = 0.040), a gene associated with GTP binding (*ENSBTAG00000039928*, FC = -71.25, *P* = 0.011), gamma-glutamyltransferase 5 (*GGT5*, FC = -68.65, *P* = 0.006), matrix metallopeptidase 11 (*MMP11*, FC = -43.94, *P* = 0.041), and glycine decarboxylase (*GLDC*, FC = -29.47, *P* = 0.049). These genes were all downregulated in the R calves.

There were 10 genes that were differentially expressed between at least 2 tissues (Table 3.4). These shared genes tended to have smaller difference in expression level, falling around 1.8 to 2.3 times more or less expressed.

4.3 *Functional annotation and pathway analyses*

Functional annotation was conducted using PANTHER for differentially expressed genes for each tissue. For all three, the majority of genes were associated with cellular processes or metabolic processes (Table 3.5). In adipose, 26.7% genes were associated with metabolic processes and 22.1% genes were associated with cellular processes. In LD, 81 genes (34.0%) were associated with metabolic processes and 73 genes (30.7%) were associated with cellular processes (Table 3.5). In liver, 32.0% genes were associated with metabolic processes and 26.0% genes were associated with cellular processes (Table 3.5).

Pathway analysis was conducted using KEGG (Appendix A) for differentially expressed genes among tissues. Pathways that had < 3 genes involved within each tissue were ignored. There were no specific pathways that involved 3 or more genes in liver; therefore, no KEGG pathway analysis was conducted. In adipose, the pathways that

involved the most genes were carbon metabolism (n = 13), protein digestion and absorption (n = 9), biosynthesis of amino acids (n = 8), and PPAR signaling pathway (n = 8). In LD, the pathways that involved the most genes were oxidative phosphorylation (n = 17), ribosome (n = 15), carbon metabolism (n = 6), and PI3K-Akt signaling pathway (n = 4).

5. **Discussion**

Because of the increasing cost of the protein used in milk replacer, it is important for producers to know what diet is most suitable for their farm. While feeding animals more feed or more energy can be beneficial for the animal, the producer needs to find the ideal level of nutrition to prevent obesity. The calf should also efficiently convert and partition that energy to meet their biological needs. In 2016, the price of milk whey protein increased from $0.58/lb in January to $0.87/lb in December (B. Gould, 2017). However, this early investment in proper calf nutrition could prevent the producer from losing money further in the cow's life. We observed that E calves had an increased differential gene expression in LD, adipose, and liver tissues. This increased dietary energy appeared to serve as a catalyst for increased gene expression that resulted in the observed increases in tissue and calf growth.

The differentially expressed genes in tissues primarily related to growth (LD and adipose) were primarily metabolism genes. In LD, the differentially expressed genes indicated that R calf tissues could be undergoing oxidative stress. All of the differentially expressed oxidative phosphorylation genes were involved in either the NADH dehydrogenase complex, cytochrome c oxidase, or ATP synthase. Genes involved in NADH dehydrogenase (i.e., *ND1*, *ND2*, *ND4L*) were all down-regulated in R calves. This

complex generates the majority of the proton gradient for the oxidative phosphorylation process (Sazanov and Hinchliffe, 2006). Cytochrome c oxidase also helps generate this proton gradient, and genes assisting in this process (*COX1*, *COX3*) were down-regulated in R calves as well. This proton gradient is necessary to generate ATP using ATP synthase, so a down-regulation of these genes could suggest that less ATP is generated in the mitochondria of LD cells. Subsequently, there was also down-regulation of genes involved in ATP synthase in R calves, specifically those that convert ADP to ATP in the F1 unit (i.e. *ATP5A1*, *ATP6*, *ATP8*; Sazanov and Hinchliffe, 2006). This could be a result the interrelationship between genes involved in generating the proton gradient with genes associated with ATP synthase. If less protons are available to pass through the channel, there is not a need to increase expression of genes involved in generating ATP (Berg et al., 2002).

It is possible that the decreased dietary energy in R calf diets could have exposed them to chronic stress. A key stress response pathway is the p53 signaling pathway, a stress response pathway involving the tumor suppressor protein p53. These stress signals activate the p53 protein to behave as a transcription factor to initiate cell cycle arrest, apoptosis, or cellular senescence (Harris et al., 2005). One differentially expressed gene in the p53 signaling pathway that was down-regulated in R calves was *cyclin dependent kinase inhibitor 1A* (***CDKN1A***), which plays a key role in cell cycle regulation and apoptosis (UniProt, 2017). It tends to be up-regulated during periods of stress, such as amino acid starvation, exposure to radiation, DNA damage response, and states of hypoxia (UniProt, 2017). The high levels of expression of *CDKN1A* in the LD in R calves could indicate that the calves are undergoing a period of subacute stress. As the

calves start to experience stress from the lack of amino acids in the diet, the possibility of damage to the DNA increases. The increased DNA damage signals the expression of *CDKN1A* to prevent the cells with damage from passing on the mutated genetic material. The adverse effect would come from the continued expression of *CDKN1A*, where fewer cells are experiencing hypertrophy, decreasing the size of the muscle.

Another gene involved in the p53 signaling pathway that was down-regulated in R calves was *growth arrest and DNA damage inducible gamma* (***GADD45G***). This gene acts similarly to *CDKN1A* in that it is expressed to regulate cell proliferation in response to stress, but *GADD45G* increases apoptosis, or programmed cell death (UniProt, 2017).

Finally, in LD of R calves *sestrin 1* (***SESN1***), a cellular response protein that is signaled by the p53 tumor suppressor protein, was also down-regulated (UniProt, 2017). Gene *SESN1* is signaled by p53 when there is DNA damage oxidative stress and is co-expressed with *GADD45G*. It is possible that due to oxidative stress and the need to repair damaged DNA, the LD cells in R calves responded by decreasing cell proliferation to prevent more DNA damage, increasing apoptosis to destroy the cells with damaged DNA, and increased expression of protein to regulate DNA repair. While it is unclear where the damage to the DNA originated from, but it is possible that oxidative stress could have led to mitochondrial DNA damage (Yakes and Van Houten, 1997). The damage to mtDNA take longer to repair than nuclear DNA damage, so extended period of oxidative stress could exacerbate this effect.

In adipose, the metabolic pathway that seemed to have the greatest change in gene expression with change in diet was the peroxisome proliferator-activated receptor (PPAR) signaling pathway. In ruminants, this pathway is involved in lipid metabolism,

anti-inflammatory response, and growth and development (Bionaz et al., 2013). The differential expression of these genes makes sense in these calves, as the change in dietary energy could lead to a change in the magnitude of lipid metabolism. In this pathway, the majority of genes that were differentially expressed were involved in fatty acid oxidation (i.e. acyl-CoA Dehydrogenase, medium chain specific (*ACADM)*, carnitine palmitoyltransferase 1A (*CPT1A*), peroxisomal acyl-coenzyme A (*ACOX1*)) or fatty acid transport acyl-CoA synthetase long-chain family members 1 and 4 (*ACSL1*, *ACSL4*)). We observed that these genes were all up-regulated in R calves. This could be because R calves needed to metabolize fatty acids from fat stores more than E calves in order to use the energy for other physiological processes such as growth. Another reason that these appear up-regulated in R calves could be that the products of these genes were already highly produced in E calves and therefore these genes have no reason to be expressed. An analysis of the products of these genes would need to be performed to confirm the reason for this gene regulation.

Another gene that was down-regulated in R calves was phosphoenolpyruvate carboxykinase 2 (*PCK2*), which is a rate-limiting step in gluconeogenesis responsible for the generation of phosphoenol-pyruvate. As a calf matures, there is an increase in gluconeogenesis and decrease in glycolysis in LD, liver and adipose (Howarth et al., 1968). This possible reduction in gluconeogenesis in R calves could be an indication of decreased development of the liver, LD, and adipose. While gluconeogenesis does not normally occur in adipose tissue, it can occur in the cortex of the kidneys (Gerich et al., 2001). Because adipose tissue samples were collected from around the kidney, it is possible for some of the differentially expressed genes to be associated with kidney

function. In humans, the cortex of the kidney significantly contributes to total gluconeogenesis in the body (Gerich et al., 2001). The kidney was previously thought to not undergo this process except during periods of starvation or acidosis, but that has since been proven to not always be the case (Joseph et al., 2000; Meyer et al., 2002). As the calves develop, the production of glucose in their kidneys could increase. Gluconeogenesis genes could be up-regulated in E calves because they grew at a faster rate and the kidneys were more larger than R calves. But, an increase in size is not directly indicative of an increase in development. To confirm this, future studies would need to analyze the gluconeogenesis genes expressed in the cortex of the kidney, kidney adipose, and subcutaneous adipose in calves on different diets.

Finally, oxidative stress may also be linked to hyperglycemia and diabetic response. Diabetes is usually associated with an impaired antioxidant capacity, which could allow for reactive oxygen species to affect cells (Rolo et al., 2006). Hyperglycemia can damage cells, and one of the key pathways associated with hyperglycemia is advanced glycation end product (**AGE**) formation (Rolo er al., 2006). The production of these AGE can lead to inflammation, thrombogenesis (formation of a clot), and mesangial matrix expansion (KEGG, 2014). While we would expect a high level of genes associated with AGE production in R calves, these genes were down-regulated. The increased gene expression of genes related to the AGE response (i.e. *ICAM1*, *CCL2*, *FN1*) in adipose in E calves indicates that the diet could be inducing a diabetic-like response. While high energy will increase the rate of growth for the calves, if too much energy or fat is in the diet, this may lead to complications. Though E calves did not elicit an apparent diabetic response, it is possible to generate this response if energy levels are

too great. Therefore, the ideal energy level may reside somewhere between the R and E diets. Hyperglycemia also leads to an increase in the conversion of glucose to sorbitol, which leads to decreases in NAPDH and glutathione (Brownlee, 2001). There are some glutathione metabolism genes (i.e. *GGT5*, *GPX3*) in the liver that are down-regulated in R calves. The R calves appeared to be experiencing oxidative stress and hyperglycemia on the cellular level. There should be a decrease in glutathione and simultaneously a decrease in the expression of genes needed for its metabolism. In order to examine if the R calves are experiencing hyperglycemia, further analysis of the level of glutathione as well as AGE in the adipose and liver will need to be examined.

Multiple genes associated with cell cycle, cell proliferation, and apoptosis were differentially expressed in both the adipose and LD. In LD, there was an up-regulation *CDKNIA*, a gene responsible for cell cycle arrest, in R calves by a magnitude of 10.40. There was also an up-regulation of *GADD45A* in LD in R calves, which is a gene that promotes cell cycle arrest as well. The up-regulation of these genes in R calves could explain the decreased carcass weight, as the skeletal muscle cells would not proliferate. This also could explain the observed increases in ADG and G:F in E calves. As the E calves were ingesting more energy, their adipose and LD cells were proliferating at an increased rate each day.

6 **Conclusion**

Overall, we conclude that the increase in dietary energy led to differential gene expression in tissues related to growth and metabolism. Of the 505 differentially expressed genes, 238 genes were found in adipose tissue, 227 genes were found in LD tissue, and 40 genes were found in liver tissue. A large amount of differentially expressed

genes in LD were associated with oxidative phosphorylation. Down-regulation of these genes in R calves could be a possible indicator of oxidative stress. In adipose, genes associated with gluconeogenesis were upregulated in E calves, which suggests that greater energy was being mobilized for growth and development. Genes associated with cell cycle arrest were up-regulated in R calves in both adipose and LD tissues. This could be a possible explanation for increase in weights of tissues in E calves, as well as the increase of ADG and G:F in E calves. According to these gene expression profiles, E calves appeared to produce increased levels of ATP through oxidative phosphorylation. The increase in genes associated with gluconeogenesis and decrease in genes associated with glycolysis in E calves suggests that these calves were able to produce more energy for growth from their diet compared to R calves. In order to confirm the gene expression, protein expression profiles need to be analyzed.

8. Figures and Tables

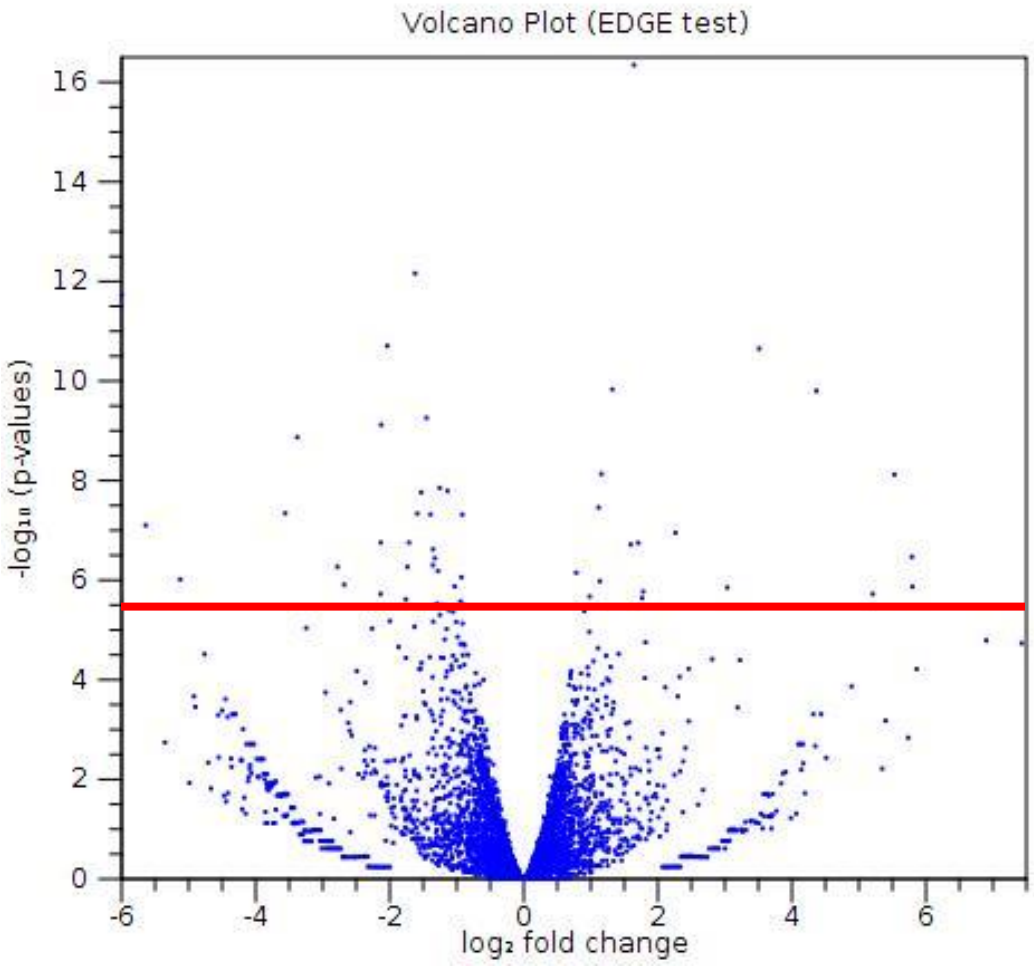

Figure 3.1.Volcano plot comparing the expression level of genes between E and R calves' adipose tissue. These depict the magnitude of fold change (FC), or differential expression, on the x-axis as well as the p-value for the level of FC between the two groups of calves on the y-axis. The value of FC is in reference to the level of expression of a gene in the R calves in relation to the E calves. For example, GADD45A has a FC of 1.573, meaning that R calves expressed this gene 1.573 times more than E calves. Points above the red line have a FDR p-value < 0.05.

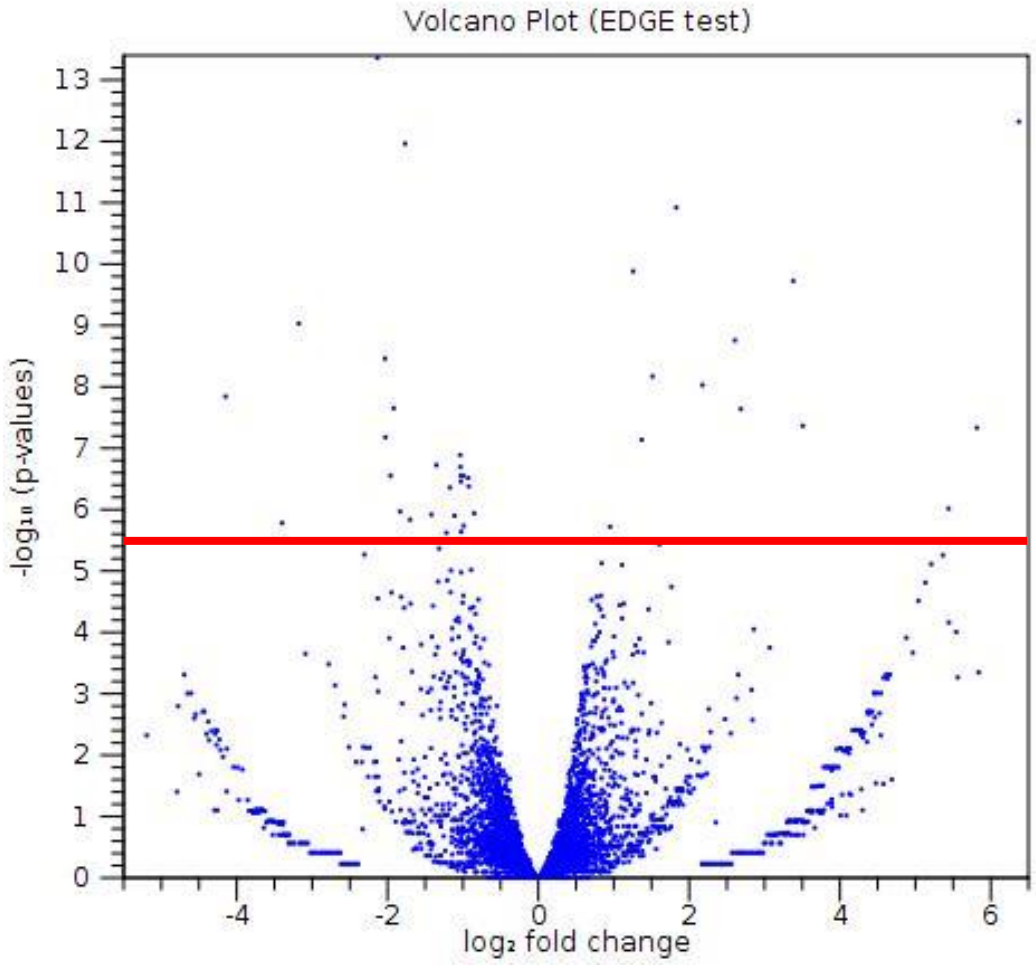

Figure 3.2 Volcano plot comparing the expression level of genes between E and R calves' LD tissue. These depict the magnitude of fold change (FC), or differential expression, on the x-axis as well as the p-value for the level of FC between the two groups of calves on the y-axis. The value of FC is in reference to the level of expression of a gene in the R calves in relation to the E calves. For example, GADD45A has a FC of 1.573, meaning that R calves expressed this gene 1.573 times more than E calves. Points above the red line have a FDR p-value < 0.05.

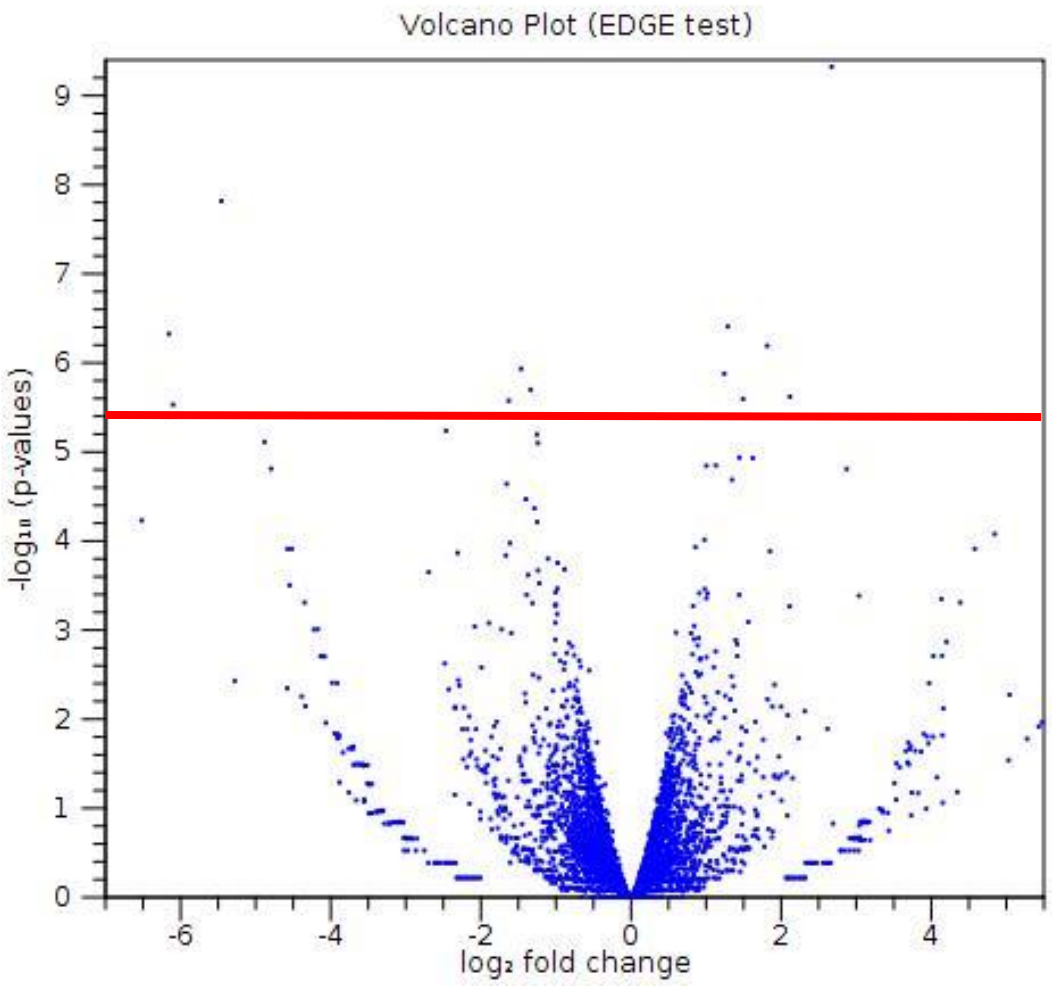

Figure 3.3 Volcano plot comparing the expression level of genes between E and R calves' liver tissue. These depict the magnitude of fold change (FC), or differential expression, on the x-axis as well as the p-value for the level of FC between the two groups of calves on the y-axis. The value of FC is in reference to the level of expression of a gene in the R calves in relation to the E calves. For example, GADD45A has a FC of 1.573, meaning that R calves expressed this gene 1.573 times more than E calves. Points above the red line have a FDR p-value < 0.05.

Table 3.1 RNA integrity values (RIN) for the samples that underwent RNA sequencing. A RIN of 7 was set as the threshold for samples.

Calf	Diet	Adipose	LD	Liver
19030	E[1]	7.3	8.6	8.3
19032	R[2]	9.6	7.2	8
19033	E[1]	9	N/A	9
19034	R[2]	9.6	7.7	7.2
19035	R[2]	9.5	N/A	8.4
19036	R[2]	9.5	7.9	7.1
19043	E[1]	8.3	7.5	8.7
19051	R[2]	9.4	7.8	8.7
19052	E[1]	9.8	7.6	8.1
3260	E[1]	9	8	7.1

1. Enhanced (E): 28% CP, 25% Fat.

2. Restricted (R): 20% CP, 20% Fat.

Table 3.2 Average daily gain (ADG), gain to feed ratio (G:F), and weights taken at time of harvest for calves either the Enhanced (E)[1] or Restricted (R)[2] diet.

Item	R	E	SEM	P-value
ADG, kg	0.22	0.76	0.06	0.007
G:F, kg	0.06	0.10	0.01	< 0.001
Carcass, kg[3]	48.6	77.6	1.10	< 0.05
Liver, kg[3]	0.94	1.79	0.05	< 0.05
Liver, g/kg BW[3]	1.94	2.32	0.10	< 0.05
Kidney Average, kg[3]	17	19	0.02	< 0.05
Kidney Average, g/kg BW[3]	0.35	0.30	0.03	> 0.10

1. Enhanced (E): 28% CP, 25% Fat;

2. Restricted (R): 20% CP, 20% Fat

3. Information modified from Geiger et al. (2015)

Table 3.3 The 10 genes with the greatest magnitude of fold change (FC) in each tissue.

Adipose			Longissimus Dorsi			Liver		
Gene	FC	*FDR-corrected P-value*	Gene	FC	*FDR-corrected P-value*	Gene	FC	*FDR-corrected P-value*
TGM3	172.39	0.003	*HIF3A*	82.89	<0.001	*OAS1X*	-91.71	0.040
ENSBTAG00000009144	119.50	0.003	*RSPO3*	57.23	0.030	*ENSBTAG00000039928*	-71.25	0.011
AMPH	-63.73	0.000	*MT3*	56.25	<0.001	*GGT5*	-68.65	0.006
TTC25	58.14	0.008	*WDR86*	47.21	0.032	*MMP11*	-43.94	0.041
KRT79	55.61	<0.001	*ENSBTAG00000025258*	46.58	0.011	*GLDC*	-29.47	0.049
KLHDC7A	55.31	<0.001	*NPTX1*	43.55	0.008	*APOA4*	28.71	<0.001
SPP2	-49.98	<0.001	*IFITM5*	43.41	<0.001	*IGFBP2*	-27.78	0.001
ENSBTAG00000048246	46.18	<0.001	*SLC9A2*	41.22	0.001	*RIBC1*	23.89	0.008
ENSBTAG00000037890	42.21	0.039	*SESN1*	37.05	0.002	*ENSBTAG00000022396*	-23.81	0.003
ENSBTAG00000015330	36.95	0.001	*FAM171A2*	35.12	0.003	*ANGPTL8*	-22.94	0.006

Table 3.4 List of genes (n=10) that are differentially expressed in at least 2 of the 3 tissue types.

	Adipose		Longisimus Dorsi		Liver	
Gene	*FC*	*FDR Corrected P-value*	*FC*	*FDR Corrected P-value*	*FC*	*FDR Corrected P-value*
GOT1	1.980	0.001	-1.919	0.003	2.719	0.036
IDH3A	2.244	0.001	-2.141	0.008	-	-
GPCPD1	1.833	0.001	-1.775	0.041	-	-
ENSBTAG00000013264	1.523	0.000	1.634	0.005	-	-
ENSBTAG00000009908	1.521	0.001	1.570	0.032	-	-
CCDC80	-1.623	0.001	-2.941	0.042	-	-
SPARC	-1.884	< 0.001	-4.473	0.032	-	-
COL15A1	-1.983	< 0.001	-1.895	0.025	-	-
GADD45A	-2.168	< 0.001	1.573	0.045	-	-
CYR61	-2.334	< 0.001	3.309	0.014	-	-
COL3A1	-2.642	< 0.001	-1.954	0.038	-	-

Table 3.5 PANTHER analysis results for each tissue. The first column represents the number of genes in each tissue that are associated with the pathway type and the second reflect the percentage those genes account for in total matched genes.

	Adipose		LD		Liver	
Process	Number of genes	Genes out of total group (%)	Number of genes	Genes out of total group (%)	Number of genes	Genes out of total group (%)
Biological Adhesion	9	4.30	3	1.60	0	0.00
Biological Regulation	32	15.40	10	5.30	4	12.50
Cellular Component Organization/Biogenesis	40	19.20	20	10.70	1	3.10
Cellular Process	93	44.70	73	39.00	13	40.60
Developmental Process	21	10.10	11	5.90	3	9.40
Immune System Process	33	15.90	6	3.20	2	12.50
Localization	24	11.50	13	7.00	3	9.40
Locomotion	3	1.40	0	0.00	1	3.10
Metabolic Process	112	53.80	81	43.30	16	50.00
Multicellular Organismal Process	19	9.10	8	4.30	2	6.30
Reproduction	1	0.50	4	2.10	1	3.10
Response to Stimulus	33	15.90	9	4.80	2	6.30

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CHAPTER 4

CONCLUSION AND IMPLICATIONS

1. Summary and Future Studies

There was a difference in the transcriptome and the microbiome of Holstein heifers fed milk replacers with different levels of dietary energy during the pre-weaning period. The R calves seemed to have a microbiome associated with younger heifers. The higher levels of Proteobacteria and Firmicutes in the microbiome of R calves is more closely associated with calves at 3 d of age. It is also possible that the higher levels of *Proteobacteria* in R calves could be eliciting an immune response due to the increased presence of *Enterobacteria.* There was in increase in expression of some genes related to immune response in the adipose tissue in R calves, but RNA information would need to be collected from rumen tissue to understand how the *Enterobacteria* are directly influencing the calf tissues.

However, in the adipose and LD tissues, the differentially expressed genes seemed to be showing signs of oxidative stress in R calves. The upregulation of genes related to oxidative phosphorylation in E calves could mean that pathway is able to generate more ATP to perform other cellular activities, such as cell proliferation. The upregulation of genes located in the p53 pathway in R calves shows that they could be responding to some stress signal, such as lack of nutrition or oxidative stress. While we only have RNA expressing information currently, analysis of the proteins present in each calf would provide more information as to why these transcripts are differentially expressed.

Further examination of the effects of different levels of dietary energy should be conducted. While this study does provide information on the immediate effects, it is possible these differences could be carried into maturity and throughout the cow's life. The change in the rumen microbial composition before weaning could lead to a different microbial composition throughout life, which could affect feed efficiency of the animal. If the change in calf diet does permanently affect the rumen microbiome, it could present an opportunity to program the calf for future production. It would also be necessary to examine how the dietary energy affects the metabolome of the rumen. This metabolome could act as a phenotype from which to examine if the calf is mature enough to be weaned. As for the transcriptome, the mechanisms controlling the differential expression of some genes needs to be characterized. While the presence of more RNA transcripts indicates more gene activity in E calves, this could be because R calves already have the products from these genes and do not need to express them at high levels.

APPENDICES

Table S1. Operational taxonomic unit (OTU) counts for each OTU that was assigned a taxonomy on the phyla level. OTUs are organized by phyla.

Phyla	TAXANAME	Enhanced	Restricted	Total
Actinobacteria				
A	actinobacterium CH9 7974	1	0	1
A	Actinoplanes violaceus; IMSNU 22136 1625	0	1	1
A	Atopobium rimae; JCM 10299 9129	3	0	3
A	Bifidobacterium merycicum; KCTC 3369 8411	44	1	45
A	Bifidobacterium merycicum; KCTC 3369 8411	44	1	45
A	Bifidobacterium pseudocatenulatum (T); JCM 1200 269	1	0	1
A	Bifidobacterium sp. DJF_WC44 591	22	13	35
A	Bifidobacterium sp. DJF_WC44 591	22	13	35
A	Bifidobacterium sp. group I-3 1181	3	3	6
A	Bifidobacterium thermacidophilum (T) 1038	0	1	1
A	Brachybacterium paraconglomeratum; TUT1011 1811	1	0	1
A	Catellatospora sp. NEAU-SH16 2045	1	0	1
A	Coriobacterium sp. CCUG 33918 4524	2	0	2
A	Dactylosporangium aurantiacum (T); DSM 43157 2690	0	1	1
A	Dermatophilus-like sp. V4.BS.12; V4.BS.12 = MM_2930 2689	1	0	1
A	Dietzia maris (T) 2481	1	0	1
A	Eggerthella lenta (T); ATCC25559 3144	0	1	1
A	Gordonibacter pamelaeae; JCM 16334 3145	0	1	1
A	Janibacter limosus (T); DSM 11140T 847	1	0	1
A	Knoellia subterranea (T); type strain: HKI 0120 = DSM 12332 = CIP 106776 1518	1	0	1
A	Kribbia sp. JL1069 2483	1	1	2

A	Microbacterium imperiale (T); DSM 20530 849	1	0	1
A	Micromonospora sp. Y10 48	1	0	1
A	Mycetocola sp. OS-74.a 3744	1	0	1
A	Nakamurella flavida (T); DS-52 9018	1	0	1
A	Nocardioides plantarum; DSM 11054T 2795	1	0	1
A	Nocardioides sp. MN12-14 2796	0	1	1
A	Okibacterium sp. Asd M5-11B 5923	1	0	1
A	Olsenella genomosp. C1; C3MLM018 8549	2	1	3
A	Olsenella sp. SK9K4 7825	139	232	371
A	Ornithinicoccus hortensis (T); KHI 0125 2791	1	0	1
A	Ornithinicoccus sp. TUT1233 2792	1	0	1
A	Propionibacteriaceae bacterium SH081 8681	1	0	1
A	uncultured Actinobacillus sp.; 402B07(oral) 740	0	1	1
A	uncultured actinobacterium; RF70 10323	0	1	1
A	uncultured Leifsonia sp.; B02-11B 2485	1	0	1
A	uncultured Microbacteriaceae bacterium; B04-04A 7663	1	0	1
A	uncultured Olsenella sp.; b4-234 8688	1	1	2
Archea				
ARC	3:334429-335902 Thermoplasmatales_archaeon_BRNA1,_complete_genome 9227	0	20	20
ARC	8:1662093-1663573 Methanobrevibacter_sp._AbM4,_complete_genome 8395	24	81	105
ARC	8:2041886-2043363 Methanobrevibacter_ruminantium_M1,_complete_genome 8803	11	3	14
ARC	Methanobrevibacter sp. 87.7 11175	6	4	10
ARC	Methanobrevibacter sp. 87.7 11175	6	4	10
ARC	Methanosphaera stadtmanae (T); DSM 3091 8912	1	2	3
ARC	uncultured archaeon; C-71 231	2	0	2
ARC	uncultured archaeon; C-72 1620	0	1	1
ARC	uncultured archaeon; D-34 8109	1	0	1
ARC	uncultured archaeon; F-2 68	0	1	1

ARC	uncultured crenarchaeote 9479	1	0	1
ARC	uncultured Methanobrevibacter sp.; 26 6701	0	6	6
ARC	uncultured Methanoplasmatales archaeon; Ana_1 9478	3	0	3
Bacteriodetes				
B	Algoriphagus terrigena (T); DS-44 2148	1	2	3
B	Alloprevotella sp. feline oral taxon 309; UI031 8124	1	0	1
B	Bacteroides coprocola; M11 6418	1	0	1
B	Bacteroides finegoldii (T); JCM 13345; 199T 593	39	1	40
B	Bacteroides finegoldii (T); JCM 13345; 199T 593	39	1	40
B	Bacteroides helcogenes; JCM6297; 15 3	1	0	1
B	Bacteroides ovatus (T); JCM 5824T 1242	5	4	9
B	Bacteroides ovatus (T); JCM 5824T 1242	5	4	9
B	Bacteroides plebeius; M14 4343	1	0	1
B	Bacteroides salyersiae; JCM12988; 7 5	2	0	2
B	Bacteroides sp. AR20 7	169	38	207
B	Bacteroides sp. AR29 10	20	29	49
B	Bacteroides sp.; BV-1 11	0	5	5
B	Candidatus Amoebinatus massiliae; CY2301169 3159	1	0	1
B	Chryseobacterium haifense; WF50 10053	3	0	3
B	Chryseobacterium solincola (T); 1YB-R12 1726	2	1	3
B	Epilithonimonas lactis (T); H1 4943	1	1	2
B	Flavobacterium rivuli (T); type strain: WB3.3-2 7208	0	1	1
B	Haloanella gallinarum; PS3 2147	16	1	17
B	Parabacteroides chinchillae; JCM 17104 6948	4	3	7
B	Parabacteroides distasonis (T); JCM 5825 1386	7	5	12
B	Parabacteroides gordonii (T); JCM 15724; MS-1 7345	1	0	1
B	Parabacteroides merdae (T); JCM 9497 6	51	17	68
B	Pedobacter aquatilis (T); type strain: AR107 5441	1	0	1

B	Pedobacter bauzanensis (T); BZ42 2611	11	1	12
B	Pedobacter piscium (T); DSM 11725T 2704	0	1	1
B	Pedobacter sp. An13 8011	1	0	1
B	Pedobacter sp. WF1 9584	2	0	2
B	Porphyromonas sp. UQD 414 4538	0	104	104
B	Porphyromonas uenonis; F0120 1243	76	0	76
B	Prevotella albensis (T); M384 1632	42	44	86
B	Prevotella brevis; BP5-19 1827	0	6	6
B	Prevotella bryantii (T); B14 (DSM 11371, species type strain) 1717	22	10	32
B	Prevotella buccalis; JCM 12246 5961	29	10	39
B	Prevotella buccalis; JCM 12246 5961	29	10	39
B	Prevotella dentasini (T); NUM 1903 1530	33	27	60
B	Prevotella genomosp. C1; C3MKM081 1824	1	0	1
B	Prevotella genomosp. C2; C3MLM058 7350	19	8	27
B	Prevotella genomosp. P6; P4PB_24 1949	13	1	14
B	Prevotella genomosp. P7 oral clone MB2_P31 3647	2	8	10
B	Prevotella genomosp. P7 oral clone MB2_P31 3647	2	8	10
B	Prevotella intermedia; ChDC KB3 1040	0	1	1
B	Prevotella marshii (T); E9.34 5643	1	0	1
B	Prevotella micans; 4D22 3876	5	4	9
B	Prevotella micans; 4D22 3876	5	4	9
B	Prevotella multisaccharivorax (T); JCM 12954 6441	19	5	24
B	Prevotella nigrescens; ChDC KB5 3510	1	1	2
B	Prevotella oralis (T); ATCC 33269 3034	17	8	25
B	Prevotella ruminicola; L16 1184	302	108	410
B	Prevotella salivae (T); JCM 12084; EPSA11 3036	1	19	20
B	Prevotella sp. BP1-56 1826	2237	19	2256
B	Prevotella sp. canine oral taxon 195; QD038 6532	3	8	11

B	Prevotella sp. canine oral taxon 226; 2A167 2057	8	372	380
B	Prevotella sp. canine oral taxon 282; ZP040 7358	0	1	1
B	Prevotella sp. canine oral taxon 284; ZP074 2236	2	2	4
B	Prevotella sp. canine oral taxon 372; 2B169 7532	0	1	1
B	Prevotella sp. oral clone AA016 5375	2	1	3
B	Prevotella sp. oral clone AH125 6751	2	1	3
B	Prevotella sp. oral clone BE073 897	84	183	267
B	Prevotella sp. oral clone FL019 1716	0	1	1
B	Prevotella sp. oral clone FW035 1182	2	0	2
B	Prevotella sp. oral clone HF050 1395	3	55	58
B	Prevotella sp. oral clone ID019 2237	0	8	8
B	Prevotella sp. oral clone IDR-CEC-0024 11353	5	2	7
B	Prevotella sp. oral clone P4PB_83 P2 11352	0	1	1
B	Prevotella sp. P2A_FAAD4 11201	11	1	12
B	Prevotella sp. R79 9411	40	0	40
B	Prevotella sp. R79 9411	40	0	40
B	Prevotella sp. Smarlab 121567 6955	2	0	2
B	Prevotella stercorea (T); CB35 7171	38	14	52
B	Prevotellaceae bacterium DJF_VR15 6961	293	230	523
B	Prevotellaceae bacterium P4P_62 P1 3385	1	1	2
B	Prevotellaceae bacterium WR041 5651	1	1	2
B	Ruminobacter amylophilus (T); DSM 1361, ATCC 29744; DSM 43089 775	3	0	3
B	Sphingobacterium sp. EQH22 5443	10	1	11
B	Sphingobacterium sp. YIM 101302 544	1	0	1
B	Tannerella forsythia; FDC 331 1526	3	0	3
B	uncultured Alistipes sp.; EMP_U8 11371	2	0	2
B	uncultured Alistipes sp.; EMP_Z14 11370	9	0	9
B	uncultured Alistipes sp.; EMP_Z14 11370	9	0	9

B	uncultured Bacteroidaceae bacterium; SL15 11488	0	1	1
B	uncultured Bacteroidales bacterium; ABXD_H22 11375	1	0	1
B	uncultured Bacteroidales bacterium; EMP_B15 11032	3	0	3
B	uncultured Bacteroidales bacterium; FecD038 7526	13	2	15
B	uncultured Bacteroidales bacterium; M_Fe_Bac11 5416	0	1	1
B	uncultured Bacteroidales bacterium; M_Fe_Bac55 6209	4	1	5
B	uncultured Bacteroidetes bacterium; D2E02 7218	1	1	2
B	uncultured Bacteroidetes bacterium; g31 6799	1	0	1
B	uncultured Bacteroidetes bacterium; HG-B01168 5434	1	0	1
B	uncultured Bacteroidetes bacterium; HG-B01195 11521	1	0	1
B	uncultured Bacteroidetes bacterium; M0011_017 9	12	12	24
B	uncultured Bacteroidetes bacterium; M0027_062 3157	0	1	1
B	uncultured Bacteroidetes bacterium; MTAC21 476	0	1	1
B	uncultured Bergeyella sp.; 602D02(oral) 6780	0	2	2
B	uncultured Cytophaga sp.; B-LO-T0_OTU13 9885	1	0	1
B	uncultured Dyadobacter sp.; DM2-136 9804	0	1	1
B	uncultured Flavobacterium sp.; SNNP_2012-37 9322	1	0	1
B	uncultured Prevotella PUS9.180 5377	0	1	1
B	uncultured Prevotella sp.; 101G12(oral) 3752	2	2	4
B	uncultured Prevotella sp.; 201D06(oral) 2138	38	33	71
B	uncultured Prevotella sp.; 201F12(oral) 5141	1	0	1
B	uncultured Prevotella sp.; 201G02(oral) 5644	4	2	6
B	uncultured Prevotella sp.; 303A09(oral) 5378	20	13	33
B	uncultured Prevotella sp.; 601D02(oral) 5376	6	3	9
B	uncultured Prevotella sp.; FecIF86 4027	16	8	24
B	uncultured Prevotella sp.; J5 4757	5	0	5
B	uncultured Prevotella sp.; L4M2 enrichment clone 196.A09 5943	817	263	1080
B	uncultured Prevotella sp.; L4M2 enrichment clone 196.G09 1837	44	22	66

B	uncultured Prevotella sp.; NRCM B1 6451	7	0	7
B	uncultured Prevotellaceae bacterium; 301H11(oral) 2804	220	626	846
B	uncultured Prevotellaceae bacterium; 702G11(oral) 6443	3	7	10
B	uncultured Prevotellaceae bacterium; 702G11(oral) 6443	3	7	10
B	uncultured Prevotellaceae bacterium; ABXD_I44 11369	2	1	3
B	uncultured Prevotellaceae bacterium; ABXD_R19 11033	4	0	4
B	uncultured Prevotellaceae bacterium; dgA-52 11027	77	69	146
B	uncultured Prevotellaceae bacterium; dgC-158 11354	1	7	8
B	uncultured Ruminobacter sp.; EMP_C38 9985	16	0	16
B	Vitellibacter sp. CC-CZW007 3276	1	0	1
Firmicutes				
F	1:1209673-1211230 Bacillus_coagulans_36D1,_complete_genome 386	1	0	1
F	Allisonella histaminiformans (T); MR2 470	16	9	25
F	Blautia hydrogenotrophica; JCM 14656 5544	1	0	1
F	Butyrivibrio fibrisolvens; C211 722	5	7	12
F	Butyrivibrio hungatei (T); JK 615 880	1	0	1
F	Catabacter hongkongensis; JCM 17853 5114	2	2	4
F	Catonella genomosp. P1 oral clone MB5_P12 5033	3	0	3
F	Christensenella minuta; YIT 12065 5112	4	5	9
F	Christensenella minuta; YIT 12065 5112	4	5	9
F	Clostridiales bacterium enrichment culture clone 06-1235251-67 10033	0	1	1
F	Clostridium alkalicellulosi (T); Z-7026 7106	0	1	1
F	Clostridium beijerinckii; JCM 7833 1890	1	0	1
F	Clostridium bolteae (T); type strain: 16351 466	8	9	17
F	Clostridium indolis (T); DSM 755 1157	1	2	3
F	Clostridium islandicum; AK1 6374	0	1	1
F	Clostridium leptum; 10900 883	0	2	2
F	Clostridium piliforme; pRT 3094	1	0	1

F	Clostridium sp. BPY5 6326	0	1	1
F	Clostridium sp. NML 04A032 9458	1	0	1
F	Clostridium sp. SY8519 1065	4	9	13
F	Dialister invisus (T); E7.25 199	0	3	3
F	Dialister sp. oral clone MCE7_134 1692	0	3	3
F	Eubacterium cellulosolvens (T); ATCC 43171 790	3	4	7
F	Eubacterium sp. F1 7372	3	10	13
F	Eubacterium sp. oral clone GI038 919	0	1	1
F	Eubacterium sp. oral clone JS001 3247	2	1	3
F	Firmicutes oral clone CK030 749	3	2	5
F	Firmicutes oral clone MCE7_107 3626	6	10	16
F	Fusobacterium necrophorum subsp. null; B; FnS-40 4556	1	2	3
F	Howardella ureilytica (T); GPC 589 1155	11	17	28
F	Hungatella hathewayi 5526	22	6	28
F	Lachnospiraceae oral clone MCE10_236 2554	1	0	1
F	Megasphaera cerevisiae; JCM 6129 1374	1	2	3
F	Mitsuokella jalaludinii (T); M9 457	425	108	533
F	Oscillibacter valericigenes (T); Sjm18-20 (= NBRC 101213) 1216	0	2	2
F	Oscillospira guilliermondii; OSC4 11613	13	13	26
F	Pectinatus frisingensis; CCM 6217 1926	1	0	1
F	Phascolarctobacterium faecium 561	4	1	5
F	Planomicrobium psychrophilum (T); CMS 53or (Type strain) 369	1	0	1
F	Pseudoflavonifractor capillosus (T); ATCC 29799 2780	0	1	1
F	Ruminococcus albus 2870	46	92	138
F	Ruminococcus albus; B199 9625	0	9	9
F	Ruminococcus albus; B199 9625	0	9	9
F	Ruminococcus bromii 1070	9	5	14
F	Ruminococcus callidus 9201	1	13	14

F	Ruminococcus flavefaciens; 007; LRC017 2015	20	8	28
F	Ruminococcus flavefaciens; JM1 2192	804	316	1120
F	Ruminococcus flavefaciens; NJ 7938	1	1	2
F	Schwartzia succinivorans (T); DSM 10502T 1300	59	98	157
F	Selenomonas bovis (T); WG 341	1727	1755	3482
F	Selenomonas genomosp. P5; P4PA_145 423	8	8	16
F	Selenomonas noxia (T); ATCC 43541 1075	0	1	1
F	Selenomonas ruminantium; 65 1029	38	40	78
F	Selenomonas ruminantium; L1 329	24	84	108
F	Selenomonas ruminantium; L14 87	1	1	2
F	Selenomonas sp. oral clone AJ036 799	2	0	2
F	Selenomonas sp. oral clone FT050 891	1	0	1
F	Selenomonas sp. oral clone JI021 522	3	9	12
F	Selenomonas sp. oral clone P4PA_36 P2 9010	2	0	2
F	Shuttleworthia satelles (T); D143K-13 1480	1	0	1
F	Sporosarcina globispora; E20 140	3	0	3
F	Streptococcus sp. 13-1151-1 828	1	0	1
F	Streptococcus suis; EA1832.92 689	2	2	4
F	Streptococcus suis; ZP005 2358	0	1	1
F	uncultured Acetivibrio sp.; FecI098 4487	1	6	7
F	uncultured Bacilli bacterium; dgA-6 11456	2	2	4
F	uncultured Clostridia bacterium; dgC-100 11127	1	0	1
F	uncultured Clostridia bacterium; dgD-113 10841	3	0	3
F	uncultured Clostridiaceae bacterium; dgA-101 2197	1	0	1
F	uncultured Clostridiaceae bacterium; dgA-54 1915	72	41	113
F	uncultured Clostridiaceae bacterium; dgB-70 7283	14	5	19
F	uncultured Clostridiales bacterium; 182 3851	1	22	23
F	uncultured Clostridiales bacterium; 748 8386	0	7	7

F	uncultured Clostridiales bacterium; MgMjD-023 11467	1	0	1
F	uncultured Clostridium sp.; 16IIISN 5057	2	0	2
F	uncultured Clostridium sp.; B17_BAC_TAD 5600	0	1	1
F	uncultured Dehalobacterium sp.; 4-CP-Fe-OTU4 6405	1	0	1
F	uncultured Enterococcus sp.; Fxy007 3221	22	40	62
F	uncultured eubacterium AA08 9332	0	8	8
F	uncultured eubacterium AA14 9168	0	1	1
F	uncultured Eubacterium sp.; 12a 4658	47	37	84
F	uncultured eubacterium WCHB1-40 10375	1	0	1
F	uncultured eubacterium; 81 263	1	0	1
F	uncultured Firmicutes bacterium; BC_COM559 8528	2	7	9
F	uncultured Firmicutes bacterium; Cf4-88 7952	0	2	2
F	uncultured Firmicutes bacterium; M0011_059 5548	5	1	6
F	uncultured Firmicutes bacterium; M0014_123 1350	7	4	11
F	uncultured Firmicutes bacterium; M0014_123 1350	7	4	11
F	uncultured Firmicutes bacterium; M0027_058 5042	1	1	2
F	uncultured Firmicutes bacterium; M0027_107 3705	2	0	2
F	uncultured Firmicutes bacterium; M3-1 7083	0	1	1
F	uncultured Firmicutes bacterium; TS-42-4 1173	1	0	1
F	uncultured Lachnospiraceae bacterium; A02-04F 5528	0	1	1
F	uncultured Lachnospiraceae bacterium; FecI012 4484	1	1	2
F	uncultured Lachnospiraceae bacterium; oral taxon JKAS-021 3224	20	10	30
F	uncultured Lachnospiraceae bacterium; Rs3-9 9844	2	1	3
F	uncultured Lachnospiraceae bacterium; SL76 9940	0	1	1
F	uncultured Lactobacillus sp.; FecI084 5873	13	21	34
F	uncultured Megasphaera sp.; 31c 3133	2	5	7
F	uncultured Megasphaera sp.; L4M2 enrichment clone 196.C01 375	204	159	363
F	uncultured Peptococcaceae bacterium; L-32 6163	19	15	34

F	uncultured Ruminococcaceae bacterium; EMP_AF18 11770	0	8	8
F	uncultured Ruminococcaceae bacterium; EMP_C8 11155	0	1	1
F	uncultured Ruminococcaceae bacterium; EMP_T40 10494	0	4	4
F	uncultured Ruminococcaceae bacterium; SL109 10503	1	0	1
F	uncultured Schwartzia sp.; FecI071 4508	7	1	8
F	uncultured Selenomonas sp.; 303E07(oral) 5338	1	0	1
F	uncultured Selenomonas sp.; J7 523	10	23	33
F	uncultured Selenomonas sp.; OCH033 396	0	45	45
F	uncultured Selenomonas sp.; OCH033 396	0	45	45
F	uncultured Veillonellaceae bacterium; dgB-66 8792	1	1	2
F	uncultured Veillonellaceae bacterium; N135 81	8	12	20
F	uncultured Veillonellaceae bacterium; SGYA409 9860	1	0	1
F	uncultured Veillonellaceae bacterium; SGYA733 3630	1	0	1
Proteobacteria				
P	Achromobacter xylosoxidans subsp. null; M-4 228	0	1	1
P	Acidovorax sp. KSP2 572	1	0	1
P	Acidovorax sp. LW1 1252	2	0	2
P	Acidovorax sp. OS-6 387	0	1	1
P	Acinetobacter baumannii; KF714 187	0	37	37
P	Acinetobacter baumannii; KF714 187	0	37	37
P	Acinetobacter lwoffii; A382 389	6	0	6
P	Acinetobacter seohaensis; SW-100 323	0	41	41
P	Acinetobacter seohaensis; SW-100 323	0	41	41
P	Acinetobacter sp. RUH53T; RUH53T (Aci 694) 57	0	1	1
P	Alysiella filiformis; IAM 14895 2942	4	3	7
P	Anaerobiospirillum sp. 3J102 11263	0	1	1
P	Avibacterium paragallinarum; Modesto 1008	1	1	2
P	Bibersteinia trehalosi; PH252 = NCTC 10641 741	5	8	13

P	Bisgaard Taxon 10; 53665-03 861	1	2	3
P	Bradyrhizobium sp. ORS199 4770	1	0	1
P	Brevundimonas diminuta (T); IAM 12691 8583	1	0	1
P	Brevundimonas sp. VTT E-052914 9660	2	0	2
P	Burkholderia sp. C3B1M 1139	1	0	1
P	Campylobacter jejuni; TGH9011(ATCC43431) 2263	2	5	7
P	Campylobacter lanienae; FK171 9158	21	64	85
P	Campylobacter sp. MIT 99-7217 7424	0	1	1
P	Campylobacter sp. oral clone HB035 2952	4	1	5
P	Caulobacter endosymbiont of Tetranychus urticae; pAJ255 10354	0	1	1
P	Cellvibrio fibrivorans (T); R4079 1268	2	0	2
P	Chelatococcus asaccharovorans; CP141b 6804	1	0	1
p	Comamonadaceae bacterium MWH55 546	1	0	1
P	Comamonas sp. MBIC3885; E6 2337	0	1	1
P	Comamonas sp. SMCC B0630 173	4	6	10
P	Comamonas sp. SMCC B0630 173	4	6	10
P	Conchiformibius steedae (T); IAM 14972 4802	1	0	1
P	Delftia sp. EK3 731	7	3	10
P	Delftia sp. EK3 731	7	3	10
P	Desulfovibrio desulfuricans subsp. desulfuricans; MB; ATCC27774 494	1	0	1
P	Desulfovibrio piger (T); ATCC29098 677	250	116	366
P	Desulfovibrio sp. canine oral taxon 070; OF078 705	4	2	6
P	Desulfovibrio sp. D4 676	62	71	133
P	Desulfovibrio sp.; KRS1 527	4	1	5
P	Devosia sp. Asd M4A1 9141	3	0	3
P	Eikenella sp. canine oral taxon 049; OB066 1097	0	2	2
P	Escherichia coli 85	9	1	10
P	Escherichia coli 85	9	1	10

P	Escherichia coli; MPEC EX-33 400	1	1	2
P	Gallibacterium anatis (T); F 149 767	54	230	284
P	Haemophilus ducreyi; ATCC 33921 617	0	1	1
P	Haemophilus influenzae; M11102 136	2	0	2
P	Haemophilus parasuis; 322 119	1	0	1
P	Haemophilus parasuis; WB25/06-3 3183	0	1	1
P	Haemophilus quentini; MCCM 02026 955	1	0	1
P	Hydrogenophaga pseudoflava (T); ATCC 33668 941	1	1	2
P	Ignatzschineria larvae; L1/58 510	2	0	2
P	Kingella kingae; C01-2424 1259	0	1	1
P	Kluyvera cryocrescens (T); ATCC33435 428	1	0	1
P	Limnobacter litoralis (T); KP1-19 2940	1	0	1
P	Luteimonas composti (T); CC-YY255 908	22	4	26
P	Luteimonas sp. Q-1 2343	1	0	1
P	Lysobacter ximonensis (T); XM415 9522	0	1	1
P	Macromonas bipunctata (T); IAM 14880 377	3	0	3
P	Mannheimia granulomatis; W4672/1 2533	2	1	3
P	Mannheimia haemolytica CCUG 28148 380	2	9	11
P	Mannheimia haemolytica; PH2, PH8 599	1	2	3
P	Mannheimia haemolytica; PH704 9354	1	2	3
P	Massilia aurea (T); type strain:AP13 3280	0	1	1
P	Methylobacterium populi (T); BJ001; ATCC BAA-705; NCIMB 13946 851	1	0	1
P	Microvirga zambiensis (T); WSM3693 8148	1	0	1
P	Neisseria canis; VA25810gw_03 10173	0	2	2
P	Neisseria gonorrhoeae (T); NCTC 83785 431	2	1	3
P	Neisseria sp. canine oral taxon 268; ZN028 1096	1	1	2
P	Neisseria sp. J01 2156	0	1	1
P	Neisseria weaveri; VA6362grgr_2001 738	7	6	13

P	Oxalicibacterium flavum (T); TA17; NEU98; LMG21571 7038	1	0	1
P	Pasteurella multocida; 5 600	0	3	3
P	Pasteurella pneumotropica; CNP 160 4613	0	1	1
P	Pasteurellaceae bacterium feline oral taxon 358; 7117 10662	1	1	2
P	Pectobacterium carotovorum (T); DSM 30168 414	1	0	1
P	Phocoenobacter uteri (T); M1063U = NCTC 12872 1051	1	1	2
P	Polynucleobacter necessarius subsp. asymbioticus; QLW-P1FAT50C-4 899	2	0	2
P	Pseudomonas aeruginosa; 22 22	1	14	15
P	Pseudomonas marginalis (T); LMG 2210T (type strain; pathovar reference strain) 300	2	0	2
P	Pseudomonas putida (T); IAM 1236 338	2	0	2
P	Pseudomonas sp. GPTSA13 1275	2	1	3
P	Pseudomonas sp. SSCS3 1270	2	1	3
P	Pseudomonas sp.; OLB-1 153	1	0	1
P	Pseudomonas stutzeri; M14C 864	2	0	2
P	Pseudoxanthomonas dokdonensis (T); DS-16 868	1	0	1
P	Pseudoxanthomonas sp. A06 10671	1	0	1
P	Rhizobium sp. LXD30 8940	1	0	1
P	Rhizobium sp. PF-M 1857	1	0	1
P	Simonsiella sp. ATCC 29465; ATCC 29465; ICPB 3648 1045	1	0	1
P	Sphingomonas japonica (T); KC7 8456	1	0	1
P	Sphingomonas phyllosphaerae; FA1 8299	1	0	1
P	Sphingomonas sp. eh2 8303	0	1	1
P	Stenotrophomonas koreensis (T); TR6-01 2537	2	1	3
P	Stenotrophomonas maltophilia; HK40 46	1	0	1
P	Stenotrophomonas sp. KC-5 4632	1	0	1
P	Succinatimonas hippei (T); YIT 12066 8743	0	4	4
P	Succinimonas amylolytica (T); DSM 2873 6044	1	0	1
P	Succinivibrio dextrinosolvens (T); DSM 3072 7886	8	2	10

P	Succinivibrio dextrinosolvens (T); DSM 3072 7886	8	2	10
P	Terrahaemophilus aromaticivorans; 127W 589	0	2	2
P	uncultured alpha proteobacterium; Dolo_28 10440	1	0	1
P	uncultured alpha proteobacterium; g60 346	1	0	1
P	uncultured alpha proteobacterium; NLS2.39 11718	0	1	1
P	uncultured Aquicella sp.; Plot17-2C06 8175	1	0	1
P	uncultured beta proteobacterium; NE83 774	0	1	1
P	uncultured Campylobacter sp.; 102B05(oral) 4403	0	1	1
P	uncultured Campylobacteraceae bacterium; SHTP728 253	1	0	1
P	uncultured Comamonas sp.; KL-11-1-9 1093	106	113	219
P	uncultured Desulfovibrio sp.; ImrTc_2 9349	4	5	9
P	uncultured Desulfovibrio sp.; L4M2 enrichment clone 196.B04 2623	88	75	163
P	uncultured Desulfovibrionaceae bacterium; 290cost002-P3L-532 9046	0	1	1
P	uncultured Desulfovibrionaceae bacterium; dgC-139 675	12	19	31
P	uncultured Desulfovibrionaceae bacterium; SL12 2830	1	0	1
P	uncultured Gallibacterium sp.; SCYA505 954	4	5	9
P	uncultured gamma proteobacterium; ANOX-130 2842	2	0	2
P	uncultured Marinobacter sp.; Ppss_Ma270 3669	0	1	1
P	uncultured Massilia sp.; WRFC52 1196	1	0	1
P	uncultured Neisseriaceae bacterium; 502H02(oral) 3668	1	0	1
P	uncultured proteobacterium; R7C24 5244	1	0	1
P	uncultured Pseudomonadaceae bacterium; T301D2 7055	1	0	1
P	uncultured Succinivibrionaceae bacterium; SGYA591 547	5	0	5
P	uncultured Xanthomonadaceae bacterium; T302F03 4439	1	0	1
P	Xanthomonadaceae bacterium NML 03-0222 911	1	3	4
P	Xanthomonas sp. 3C_3 4429	1	1	2
P	Xanthomonas vesicatoria; XV1111 2	5	0	5
P	Xanthomonas-like sp. V4.BO.41; V4.BO.41 = MM_2937 865	2	2	4

Spirochetes				
S	Candidatus Treponema suis 9751	0	1	1
S	Spirochaeta sp.; NL1 9169	0	1	1
S	Synergistes sp. NML96A088 8858	65	202	267
S	Treponema parvum (T); OMZ833 4103	0	3	3
S	Treponema pectinovorum; OMZ831 3952	21	38	59
S	Treponema sp. canine oral taxon 207; PZ040 5020	1	0	1
S	Treponema sp. feline oral taxon 123; TE135 824	1	0	1
S	Treponema sp. oral clone JU025; Ju025 916	0	2	2
S	Treponema sp. Sy24 872	1	0	1
S	Treponema succinifaciens DSM 2489 648	0	381	381
S	Treponema zioleckii; kT 3960	8	4	12
S	uncultured Spirochaeta sp.; RF104 8322	0	2	2
S	uncultured Spirochaeta sp.; RP9-175 4832	1	0	1
S	uncultured spirochete; IE094 7750	0	1	1
S	uncultured Synergistes sp.; L4M2 enrichment clone 196.B09 3441	24	34	58
S	uncultured Treponema clone HsPySp1 3434	2	0	2
S	uncultured Treponema sp.; 101D06(oral) 1108	3	0	3
S	uncultured Treponema sp.; EMP_F10 9830	8	8	16
S	uncultured Treponema sp.; RF11 4829	3	4	7
S	uncultured Treponema sp.; RF25 6064	3	1	4
S	uncultured Treponema sp.; RF45 4445	2	0	2
S	uncultured Treponema sp.; RF52 5510	21	1	22
Other				
Tenericutes	Mycoplasma dispar; ATCC 27140 1109	0	1	1
Tenerticutes	uncultured Asteroleplasma sp.; 56b 6848	0	14	14
Verrucomicrobia	uncultured Verrucomicrobiales bacterium; B02-06F 1749	1	0	1
Cyanobacteria	uncultured Vampirovibrio sp.; FecD09 7883	102	44	146

Chloroflexi	uncultured Chloroflexi bacterium; g16 9587	0	1	1
Defferibacteres	Flexistipes sp. E3_33 1207	1	0	1
Dienococcus-Thermus	uncultured Deinococcales bacterium; A_469 4769	1	0	1
Lentisphaerae	Victivallaceae bacterium NML 080035 9733	31	19	50
		74908	**62491**	**137399**

Table S2: The expression values for the differentially expressed genes in adipose tissue. Expression was measured by reads per kilobase of transcript per million mapped reads (RPKM).

Feature ID	Fold change	FDR p-value correction
TGM3	172.39	0.003
ENSBTAG00000009144	119.50	0.003
TTC25	58.14	0.008
KRT79	55.61	<0.001
KLHDC7A	55.31	<0.001
ENSBTAG00000048246	46.18	<0.001
ENSBTAG00000037890	42.21	0.039
ENSBTAG00000015330	36.95	0.001
ENSBTAG00000038910	29.69	0.014
REEP6	21.67	0.032
AMDHD1	20.62	<0.001
ENSBTAG00000047177	20.00	0.032
SLC27A2	11.39	<0.001
UCP1	9.35	0.006
LCTL	9.13	0.027
FAM19A5	8.21	<0.001
ENSBTAG00000004836	7.02	0.005
SESN2	5.51	0.008
PPIF	5.49	0.039
ENSBTAG00000047869	5.01	0.010
SLC25A13	4.92	0.019
DRD2	4.79	<0.001
PPARGC1A	4.32	0.014
IDH2	3.52	0.003
ENSBTAG00000046095	3.50	0.010
ACLY	3.44	<0.001
THOP1	3.40	0.001
EBF2	3.27	<0.001
ENSBTAG00000038461	3.13	<0.001
ENSBTAG00000010829	3.02	<0.001
FBP2	2.98	0.040
ENSBTAG00000002046	2.88	0.041
ENSBTAG00000015258	2.67	0.005
ENSBTAG00000016032	2.54	0.022
RPRML	2.52	0.028
ENSBTAG00000038461	2.50	<0.001

RDH16	2.48	0.032
GPT2	2.45	0.050
GLCCI1	2.45	0.007
S1PR3	2.42	0.011
FAM160A1	2.36	0.010
COX7A1	2.35	0.025
NNT	2.34	0.020
SHMT2	2.34	0.005
COQ8A	2.26	0.013
IDH3A	2.24	0.035
PFKFB4	2.23	<0.001
TSPYL2	2.20	<0.001
SLC16A11	2.17	<0.001
TST	2.16	0.004
MDH1	2.14	0.021
MYOM1	2.12	0.029
CPT1A	2.08	0.015
MLF1	2.05	0.006
POPDC3	2.03	0.025
CHDH	2.01	0.039
TEF	1.98	0.039
GOT1	1.98	0.048
RPS6KA2	1.98	0.032
ACADM	1.98	0.001
ENSBTAG00000015314	1.97	0.002
ENSBTAG00000048135	1.97	0.044
ACOX1	1.95	0.009
SREBF1	1.95	0.014
COX7A2	1.94	0.032
ACADVL	1.93	0.007
ACSL4	1.91	0.020
PPP1R3B	1.89	0.037
ELOVL1	1.87	0.001
ACSL1	1.87	0.039
PKM	1.86	0.050
NUDT5	1.84	0.039
GPCPD1	1.83	0.050
NQO2	1.81	0.009
LARP6	1.80	0.014
RBM3	1.76	0.044
SUCLA2	1.74	0.021
HSPE1	1.73	0.022
ENSBTAG00000018768	1.72	<0.001

GSTK1	1.71	0.024
GBAS	1.70	0.049
LDLRAD3	1.70	0.014
ENSBTAG00000009603	1.70	0.042
ENSBTAG00000031723	1.68	0.014
RBMX2	1.67	0.033
CITED2	1.65	0.022
ATP1A1	1.63	0.008
IARS2	1.63	0.043
RPS8	1.62	0.009
ECHDC3	1.62	0.010
eef1d	1.60	0.013
GHITM	1.56	0.048
ENSBTAG00000008135	1.55	0.040
ENSBTAG00000013264	1.52	0.033
ENSBTAG00000009908	1.52	0.042
ENSBTAG00000020139	1.48	0.048
TMBIM6	-1.52	0.011
CD74	-1.53	0.039
ENSBTAG00000047739	-1.54	0.040
ENSBTAG00000015154	-1.55	0.038
ENSBTAG00000038025	-1.56	0.037
BoLA DR-ALPHA	-1.58	0.013
ADH5	-1.61	0.035
OPTN	-1.61	0.036
SDPR	-1.61	0.039
CCDC80	-1.62	0.038
SERPINH1	-1.63	0.041
TNFRSF21	-1.63	0.039
IGFBP7	-1.64	0.008
ANTXR2	-1.65	0.035
B3GALNT1	-1.65	0.030
ACER3	-1.66	0.046
BOLA-DRB3	-1.66	0.013
COPZ2	-1.71	0.030
KDELR3	-1.73	0.021
S100A10	-1.74	0.017
SYT4	-1.75	0.022
ESYT1	-1.76	0.039
ANXA1	-1.78	0.039
ECM2	-1.78	0.005
GYG1	-1.79	0.030
PMP22	-1.81	0.013

ENSBTAG00000030333	-1.82	0.014
MAOB	-1.83	0.039
COL5A1	-1.84	0.032
C10orf10	-1.85	0.030
RBPMS	-1.85	0.003
MSRB3	-1.85	0.007
RGS3	-1.86	0.003
LAMB2	-1.87	0.001
ANXA2	-1.88	0.005
SPARC	-1.88	0.001
ARSK	-1.88	0.037
RHOB	-1.89	<0.001
PTGDS	-1.89	0.021
ARL6IP5	-1.90	0.000
COL5A3	-1.92	0.001
MYOC	-1.93	0.003
COL6A2	-1.94	0.027
ENSBTAG00000012370	-1.94	0.007
NCALD	-1.95	0.016
COL15A1	-1.98	0.003
AIM1	-2.01	0.006
S100A11	-2.01	0.001
CCDC69	-2.03	0.024
TSPAN15	-2.04	0.016
GALNT16	-2.04	<0.001
NIPSNAP1	-2.07	0.022
LGALS3BP	-2.08	0.001
CPXM1	-2.08	0.016
ENSBTAG00000005574	-2.08	0.005
ENSBTAG00000007213	-2.11	0.008
SHISA4	-2.11	0.007
GADD45A	-2.17	0.001
TMSB10	-2.20	<0.001
ASGR2	-2.20	0.021
JAZF1	-2.22	0.008
ZFP36	-2.22	0.002
PCK2	-2.23	0.010
C4orf32	-2.26	0.003
S100A4	-2.30	0.005
GNAI1	-2.31	0.001
GPC3	-2.31	0.030
CYR61	-2.33	0.014
FHL1	-2.34	0.042

CD44	-2.35	0.005
THY1	-2.36	0.021
BCAT2	-2.36	0.035
CDKN2C	-2.38	0.001
ICAM1	-2.38	<0.001
NUPR1	-2.43	<0.001
SKAP1	-2.45	0.001
ADM	-2.51	<0.001
DKK3	-2.53	0.039
CRYAB	-2.55	0.001
FXYD1	-2.56	<0.001
SEMA3B	-2.56	<0.001
COL3A1	-2.56	0.010
LUM	-2.57	0.033
SERPINF1	-2.62	0.030
F3	-2.63	0.000
COL3A1	-2.64	0.005
ACSM1	-2.73	<0.001
IRF9	-2.76	0.024
RBP4	-2.80	0.023
LY6E	-2.83	0.016
XPNPEP2	-2.87	0.006
DUSP1	-2.89	<0.001
ENSBTAG00000003152	-2.91	0.007
ACSS2	-2.94	0.008
FN1	-3.01	<0.001
NOV	-3.02	0.037
TMEM176B	-3.03	0.035
ADRB2	-3.08	<0.001
IDO1	-3.09	0.002
RAB3B	-3.28	<0.001
FN1	-3.34	<0.001
THRSP	-3.38	0.005
DMPK	-3.39	0.001
COL1A2	-3.41	0.034
COL1A1	-3.44	0.035
STBD1	-3.54	0.044
NR4A1	-3.65	0.004
ADAMTS4	-3.99	0.001
ENSBTAG00000037399	-4.11	<0.001
SULF1	-4.37	<0.001
QPCT	-4.39	<0.001
CCL2	-4.40	0.001

ENSBTAG00000008793	-4.79	0.002
PCDH7	-5.14	0.012
CGREF1	-5.62	0.008
KCNMB1	-6.01	0.022
MARCH3	-6.14	0.041
S100A8	-6.41	<0.001
ENSBTAG00000014529	-6.64	0.030
S100A9	-6.87	<0.001
OTOP1	-7.77	0.016
ATF3	-9.48	0.002
ENSBTAG00000038124	-10.39	<0.001
PLXDC1	-11.77	<0.001
ENSBTAG00000000735	-18.07	0.050
RPS6KL1	-18.20	0.050
CYP39A1	-19.25	0.032
MAP3K7CL	-19.69	0.032
HIST1H4C	-20.03	0.032
HIST1H2BC	-20.38	0.032
ALDOB	-21.34	0.035
SSC5D	-21.62	0.021
ENSBTAG00000002416	-21.87	0.021
ENSBTAG00000009828	-22.65	0.030
ENSBTAG00000033515	-23.57	0.033
COL8A1	-27.12	0.005
NOL3	-29.84	0.027
ENSBTAG00000046193	-30.27	0.019
ITPKA	-34.35	<0.001
MMP19	-34.93	<0.001
SPP2	-49.98	<0.001
AMPH	-63.73	<0.001

Table S3. The expression values for the differentially expressed genes in longissimus dorsi (LD) tissue. Expression was measured by reads per kilobase of transcript per million mapped reads (RPKM).

Gene ID	Fold change	FDR p-value correction
HIF3A	82.89	<0.001
RSPO3	57.23	0.030
MT3	56.25	<0.001
WDR86	47.21	0.032
ENSBTAG00000025258	46.58	0.011
NPTX1	43.55	0.008
IFITM5	43.41	<0.001
SLC9A2	41.22	0.001
SESN1	37.05	0.002
FAM171A2	35.12	0.003
DYNLRB2	32.96	0.005
SNED1	32.90	0.005
FAH	31.25	0.018
EME2	29.45	0.013
GAREM2	25.32	0.031
TEX30	25.08	0.031
KLHL36	24.90	0.031
ME3	24.74	0.033
ENSBTAG00000017228	24.41	0.031
CISD1	23.87	0.032
YPEL1	23.33	0.045
RRAGB	22.84	0.045
MBNL2	22.71	0.045
NUDT16	22.65	0.045
RPS27A	22.59	0.045
ENSBTAG00000032764	22.29	0.045
HOXC10	22.21	0.045
RPL37A	22.06	0.045
ZDHHC12	21.87	0.045
CCDC190	11.37	<0.001
CDKN1A	10.40	<0.001
ETS2	8.39	0.016
PROCA1	7.25	0.010
KBTBD13	7.10	0.043
ENSBTAG00000003155	6.43	<0.001
ENSBTAG00000031214	6.29	0.031

ENSBTAG00000023659	6.10	<0.001
PRR32	4.52	<0.001
EIF4EBP1	3.55	<0.001
MYO1C	3.40	0.003
CYR61	3.31	0.014
HMOX1	3.04	0.001
BTG1	2.86	<0.001
RPL39	2.75	0.006
ENSBTAG00000039486	2.63	0.018
UCP2	2.58	<0.001
MAFF	2.53	0.013
NUAK1	2.51	0.047
GAS6	2.50	0.018
HIST1H1C	2.44	0.015
MRPS6	2.40	0.036
IGF2	2.39	<0.001
IGFN1	2.38	0.019
PGK1	2.27	0.041
ENSBTAG00000046307	2.21	0.036
TXNDC17	2.19	0.005
CTSF	2.16	0.002
ATP6V1C1	2.16	0.007
LIMS2	2.11	0.005
TCAP	2.04	0.041
PHKA1	2.00	0.012
PXMP2	2.00	0.021
CHRNA1	1.98	0.018
ENSBTAG00000030164	1.94	0.001
ADIRF	1.92	0.042
SMYD1	1.90	0.022
SESN1	1.87	0.022
ENSBTAG00000019253	1.85	0.024
PSMG4	1.83	0.032
HMGB2	1.83	0.041
APIP	1.82	0.030
ENSBTAG00000005349	1.81	0.007
RPS20	1.79	0.002
H1FX	1.79	0.027
TUBA8	1.77	0.004
MTURN	1.77	0.006
RPS28	1.76	0.011
ENSBTAG00000001648	1.74	0.013
FTH1	1.73	0.005

PRDX3	1.73	0.031
FBXO44	1.72	0.045
YIPF7	1.72	0.012
RPL11	1.71	0.004
CIRBP	1.70	0.036
ENSBTAG00000016093	1.69	0.027
RPL10A	1.69	0.009
ENSBTAG00000018800	1.68	0.013
PTRF	1.68	0.046
RPS29	1.66	0.048
RPL34	1.65	0.013
ENSBTAG00000038104	1.65	0.024
ENSBTAG00000013264	1.63	0.005
EEF1B2	1.63	0.016
RPS9	1.59	0.025
P2RY2	1.58	0.045
GADD45A	1.57	0.045
ENSBTAG00000009908	1.57	0.032
RPL30	1.57	0.027
ENSBTAG00000019718	1.57	0.044
RPS11	1.56	0.026
ENSBTAG00000013866	1.55	0.036
RCL1	1.55	0.031
RPLP0	1.53	0.032
ENSBTAG00000014208	1.53	0.029
FRS3	1.53	0.045
ENSBTAG00000003229	1.52	0.026
ITM2B	1.51	0.048
ENSBTAG00000001360	1.50	0.048
ENSBTAG00000031800	1.50	0.048
METTL11B	1.48	0.045
HSPA8	-1.58	0.025
IMMT	-1.64	0.023
FHOD3	-1.65	0.013
ATP1B1	-1.67	0.034
CHRND	-1.71	0.016
GSTP1	-1.72	0.031
ENSBTAG00000037991	-1.73	0.032
COL4A1	-1.73	0.042
NDUFS1	-1.73	0.038
NDUFA10	-1.73	0.032
ANKRD23	-1.74	0.005
GPI	-1.76	0.036

MRPS36	-1.76	0.028
SLC20A2	-1.76	0.045
ENSBTAG00000011963	-1.77	0.012
GPCPD1	-1.77	0.041
AGPAT5	-1.78	0.006
ARMCX3	-1.79	0.038
OGDH	-1.79	0.014
MB	-1.80	0.000
PNPLA2	-1.81	0.045
ND3	-1.82	0.005
LDB3	-1.85	0.010
ND2	-1.86	0.002
KLHL31	-1.87	0.037
RNF130	-1.87	0.023
LDB3	-1.87	0.005
ATP5G3	-1.87	0.009
COL15A1	-1.89	0.025
ND4	-1.90	<0.001
FDFT1	-1.90	0.022
COX3	-1.91	<0.001
PDE8A	-1.92	0.032
ENSBTAG00000010709	-1.94	0.023
ENSBTAG00000019701	-1.94	0.045
COL3A1	-1.95	0.038
RGS14	-1.98	0.032
COX1	-1.98	0.001
MAP2K6	-1.98	0.017
ENSBTAG00000020795	-1.99	0.004
ATP6	-1.99	<0.001
NEB	-2.00	0.023
HOMER1	-2.00	0.019
GYS1	-2.01	0.005
ALDH1A1	-2.02	0.031
ND4L	-2.03	0.001
RHOQ	-2.03	0.002
ENSBTAG00000008895	-2.03	0.021
ND1	-2.04	<0.001
PDE4B	-2.04	<0.001
ND6	-2.05	<0.001
COII	-2.05	<0.001
EPAS1	-2.07	0.008
DDO	-2.08	0.012
COQ10A	-2.09	0.007

ENSBTAG00000017071	-2.14	0.030
IDH3A	-2.14	0.008
ENSBTAG00000003275	-2.14	0.019
ATP8	-2.16	<0.001
ENSBTAG00000000745	-2.17	0.048
G3BP2	-2.17	0.041
ENSBTAG00000005339	-2.20	0.010
ATP5A1	-2.21	0.013
CHPT1	-2.23	0.002
MGC148692	-2.24	0.004
ND5	-2.25	<0.001
PADI2	-2.26	0.032
ALAS1	-2.30	0.036
GOT1	-2.31	0.003
ADSSL1	-2.33	0.001
ENSBTAG00000024605	-2.33	0.042
DAPK3	-2.37	0.041
CAMK2D	-2.39	0.031
ENSBTAG00000039555	-2.46	0.033
ENSBTAG00000006491	-2.49	0.001
CLIC5	-2.51	0.003
ETNPPL	-2.53	0.016
IP6K3	-2.56	<0.001
FOXS1	-2.58	0.019
CARNS1	-2.62	0.032
TFRC	-2.64	0.005
PRDX2	-2.66	0.012
FBXO40	-2.67	<0.001
ENSBTAG00000031573	-2.68	0.042
ENSBTAG00000037937	-2.76	0.045
P2RY1	-2.94	0.015
CCDC80	-2.94	0.042
EIF4G3	-3.19	0.030
MGC148692	-3.23	0.005
PPTC7	-3.25	<0.001
CKMT2	-3.40	<0.001
GADL1	-3.43	0.005
NDUFB5	-3.46	0.016
CISH	-3.53	0.004
MYL3	-3.56	<0.001
CARNMT1	-3.78	<0.001
ENSBTAG00000019915	-3.86	0.004
cytb	-3.89	<0.001

SPOCK2	-3.94	0.013
ENSBTAG00000038186	-4.07	<0.001
CA2	-4.09	<0.001
ENSBTAG00000012344	-4.37	0.045
CHRNE	-4.38	0.005
CCDC6	-4.39	<0.001
SPARC	-4.47	0.032
PNMT	-4.95	0.001
ALPL	-6.48	0.039
VASH1	-6.88	0.024
CHN1	-8.52	0.019
SLC16A6	-9.04	<0.001
ANKH	-10.55	<0.001
ENSBTAG00000001344	-17.74	<0.001
STYXL1	-24.24	0.045
SPSB4	-24.29	0.045
SLC25A4	-24.33	0.045
DKK2	-25.14	0.045
RPS7	-25.93	0.031

Table S4. The expression values for the differentially expressed genes in liver tissue. Expression was measured by reads per kilobase of transcript per million mapped reads (RPKM).

Feature ID	Fold Change	FDR p-value correction
APOA4	28.71	<0.001
RIBC1	23.89	0.008
MGLL	7.34	0.010
ASCL1	6.37	0.005
ENSBTAG00000039971	4.34	0.018
SFTPA1	3.61	0.024
AK4	3.52	0.003
MAGED1	3.08	0.008
PPP1R3C	2.82	0.003
GOT1	2.72	0.036
BHLHA15	2.54	0.007
SEC16B	2.44	0.042
ENSBTAG00000046257	2.37	0.038
LDHA	2.19	0.008
CLDN15	2.00	0.002
SLC13A5	1.97	0.008
SERPINI2	1.81	0.001
KCNJ15	-1.97	0.039
ENSBTAG00000037452	-2.15	0.039
CTSL2	-2.36	0.039
GPX3	-2.38	0.003
RAD21L1	-2.38	0.045
SLC13A5	-2.44	0.001
SLC17A4	-2.53	0.005
CDC42EP5	-2.64	0.007
SERPINA6	-2.76	0.002
CYP26A1	-3.06	<0.001
NGEF	-3.10	0.015
CRCP	-3.15	0.003
ENSBTAG00000034192	-3.17	0.039
GLB1L3	-4.95	0.032
TMEM25	-5.52	0.024
ANGPTL8	-22.94	0.006
ENSBTAG00000022396	-23.81	0.003
IGFBP2	-27.78	0.001
GLDC	-29.47	0.049

MMP11	-43.94	0.041
GGT5	-68.65	0.006
ENSBTAG00000039928	-71.25	0.011
OAS1X	-91.71	0.040