STRATEGIES TO IMPROVE HEALTH AND PRODUCTION OF DAIRY COWS DURING EARLY LACTATION

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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December 2018

STRATEGIES TO IMPROVE HEALTH AND PRODUCTION OF DAIRY COWS DURING EARLY LACTATION

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Cornell University 2018

The dairy industry directly impacts the socio-economic status of roughly 150 million families worldwide through its contribution to income, food security, and access to protein. One of the most challenging aspects for dairy cows is the transition between the last stage of their pregnancy into a new lactation. The ability of a cow to deal with extensive physiological changes during the late pregnancy and early postpartum periods influences the entire lactation in terms of milk yield, quality, and health status. Observational and interventional studies are key to understanding the complexity of physiological changes during this transition in dairy cows. For example, the identification of genomic regions associated with the development of hyperketonemia will enhance our ability to identify the most susceptible animals to these metabolic diseases. Supplementation of diets with rumen-protected branched-chain amino acids during early postpartum may be a functional strategy to prevent adverse effects of excessive negative energy balance. Utilization of these studies and future applied research will be crucial to ensure the sustainability of the dairy industry worldwide.

BIOGRAPHICAL SKETCH

Francisco Antonio Leal Yepes was born and raised in Bogotá, Colombia. He attended Champagnat School, from kindergarten through high school, where he played soccer every day and studied occasionally. On weekends during his childhood, he went to his father's farm to chase cows and hike through the Andes Mountains. When he was 11 years old, his grandfather brought him some guinea pigs and rabbits, so he started breeding them to sell the meat at restaurants (in order to buy soccer jerseys). It was then that he developed a great interest in animal production and veterinary medicine. After graduation from high school, he went to veterinary school at the University of Environmental and Applied Science in Bogotá and graduated in 2008. Shortly after veterinary school, he started a M.S. program in Animal Science while practicing veterinary medicine in small dairy and dual-purpose farms. He decided to pursue a graduate program outside of Colombia and contacted Dr. Daryl V. Nydam at Cornell University. After a couple of years working as a research assistant under the supervision of Drs. Wakshlag and Nydam and after several hundreds of biopsies in dairy cows with Dr. Sabine Mann, in 2013, Francisco started his Ph.D. in Animal Science at Cornell University. Additionally, Francisco completed a residency in Cornell's Ambulatory and Production Medicine Clinic from August 2016 to July 2018 under the supervision of Drs. Charles Guard, Mary Smith and Jessica McArt. He is pursuing board certification in the American College of Veterinary Preventive Medicine and aspires to be a research and clinical professor of production animal medicine.

Para Claire, María Luz, Constanza, Ana María y David, eternamente agradecido por su amor y apoyo en todo momento.

ACKNOWLEDGEMENTS

This dissertation tries to summarize my doctoral work. First I would like to acknowledge Dr. Daryl Nydam for being an encouraging, enthusiastic, supportive advisor during all this time and helping me to be a better person.

Dr. Sabine Mann taught me so many things and positively influenced my professional career with her incredible work ethic and willingness to help others.

Members of my committee have spent a tremendous amount of time and effort adding in my training. Thank you to Dr. Joseph Wakshlag for all the patience and guidance during my first steps in the lab; Dr. Thomas Overton for challenging me to always move one step forward; Dr. Heather Huson for her invaluable expertise in genetics and support; Dr. Jessica McArt for helping me to improve my grant application skills and all the life advice while we were freezing sticking our arms into cow's butts.

Thanks to Dr. Claire Zoellner for supporting and helping me in so many ways during this training program to advance in my career. I would like to thank Dr. Wolfgang Heuwieser, Charlen Ryan, Lauren Stanko Bristol, German Granados, John Leahy, Conor McCabe, Sarah Elizabeth LaCount, Allison Lawton Kerwin, Emily Schweitzer, Dr. Ben Kasl, Dr. Amy Vasquez, and Rodrigo Molano Torres for their invaluable help during this project.

I also want to thank the staff at the Cornell University Research Dairy Farm for taking care of the animals enrolled in our study. Special thanks to Balchem Corporation (New Hampton, NY), Pharmadoc (Lübeck, Germany) and TaiDoc (New Taipei City, Taiwan) for providing the rumen protected amino acids, point-of-care devices and test strips.

Thanks to the Agriculture and Food Research Initiative competitive grant no. 2012-67015-30230 from the United States Department of Agriculture National Institute of Food and Agriculture (Washington, DC) for supporting my doctoral work.

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CHAPTER 1

INTRODUCTION

The dairy industry contributes to income and food security as dairy is one of the most affordable sources of protein and other nutrients for millions of people around the globe. As the human population continues to increase, milk demand is expected to grow more than 91% worldwide while the milk price will rise just 4% between 2000 and 2050 (Havlík et al., 2015). Milk production from dairy cows is a complex process not only operationally but also within the cow. Pregnancy is a crucial process for the success of a dairy herd, and at the same time it is one of the most challenging periods for a dairy cow. Raising newborn dairy calves through the beginning of their first lactation is time consuming and labor intensive, with many obstacles. It is all worth it and becomes a rewarding practice when they start producing milk.

The capacity of a cow to overcome extensive physiological changes between the late pregnancy and early postpartum period impacts the subsequent lactation in terms of production, reproduction, and health status. Pregnant animals have an increased requirement for nutrients, not only to support the growing fetus but also all of the structures needed to maintain the pregnancy, including the uterus, placenta, and mammary gland development (Bauman and Currie, 1980). The end of pregnancy and the beginning of lactation is an interesting period full of challenges and adjustments within the organism. During onset of lactation, the metabolic rate increases drastically in the mammary gland tissue to maintain increasing milk synthesis. Almost all the

infectious and metabolic diseases present a higher incidence during the early postpartum period than any other stage during the lactation (Drackley, 1999, Duffield et al., 2009).

One of the most notorious issues during this period , which starts during the last few weeks of the pregnancy and lasts until calving, is the reduction in voluntary dry matter intake (**DMI**), particularly when the energy demand in dairy cows is doubled immediately after calving as compared to the immediate prepartum period (Drackley et al., 2001b). Moreover, genetic selection programs in the modern dairy industry that prioritize high milk yield have augmented the difference between nutrients consumed and nutrients needed, particularly during the early postpartum period (Tveit et al., 1992, Veerkamp et al., 2003). At the same time, many other factors influence DMI, such as advanced parity, body weight, environment, increased body condition score before calving, feed, and management of cows through their productive cycle (Roseler et al., 1997, McArt et al., 2013, Mann et al., 2015).

The inevitable consequence of reduction in DMI and increasing nutrient requirements between late pregnancy and early lactation is insufficient daily supply of nutrients for some tissues to perform their regular metabolic processes. This period of nutrient deficiency is commonly known as negative energy balance (**NEB**) (Figure 1.1). Thus, dairy cows orchestrate a metabolic response to counteract the lack of nutrients by breaking down body reserves such as fat and muscle and mobilizing them via the bloodstream, so the liver can use them as energy precursors through different metabolic pathways (Figure 1.2). The concentration of insulin and glucose in plasma drops drastically between late pregnancy and early postpartum activating signals that induce lipolysis and proteolysis (Ingvartsen and Andersen, 2000, Reynolds et al., 2003).

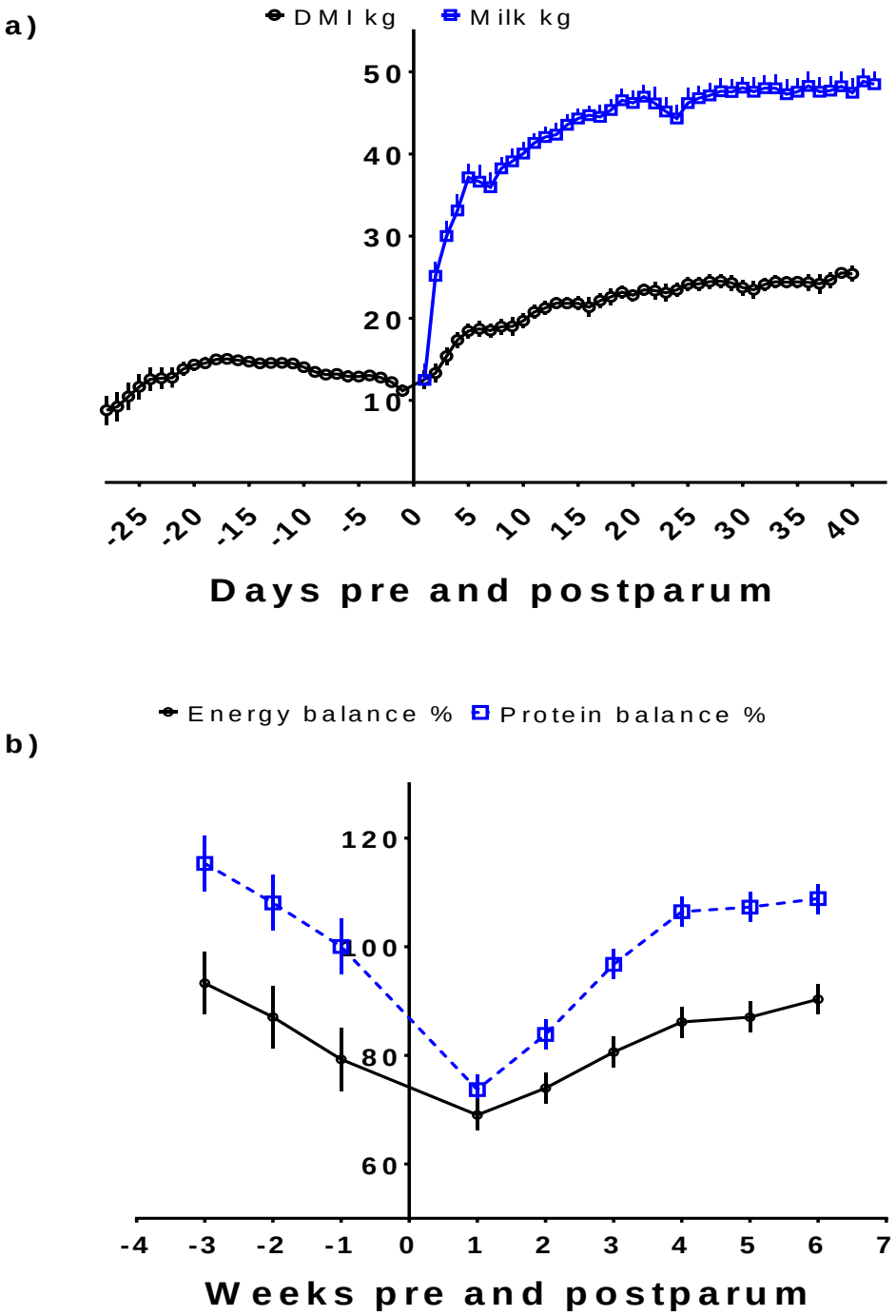

Figure 1.1. Average (95% CI) for: a) dry matter intake (kg) pre and postpartum, and daily milk yield (kg) 1 to 42 days in milk; b) energy balance and protein balance pre and postpartum calculated on CNCPS v6.55, Leal Yepes F.A. et al. (2018) unpublished.

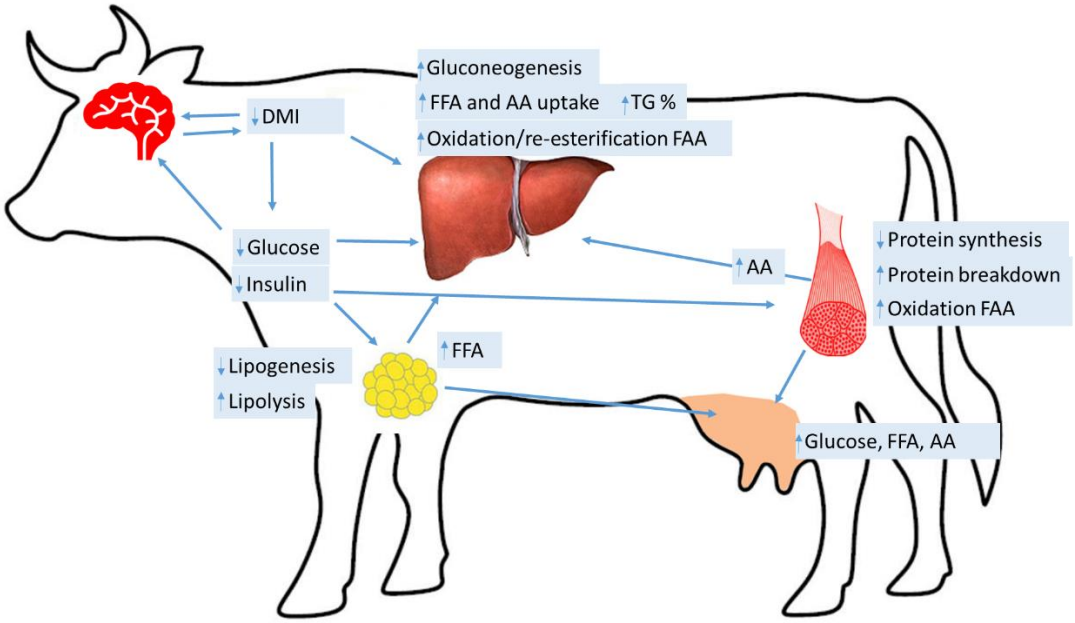

Figure 1.2. Summary of a few metabolic changes in response to a reduction in dry matter intake (DMI) during early postpartum in dairy cows, adapted from Bauman and Currie (1980), Ingvartsen and Andersen(2000), and Kuhla et al. (2011).

Few blood metabolites have been commonly used as indicators of the degree of energy reserves mobilization for example free fatty acids (**FFA**), β-hydroxybutyrate (**BHB**) and glucose (Leblanc, 2010, Ospina et al., 2013). There are three ketone bodies, acetoacetate, BHB, and acetone, of which BHB is the most stable in blood. Ketone bodies are commonly found in ruminant organisms as a consequence of the ruminal fermentation of feed material into volatile fatty acids such us butyrate (Van Soest, 1963). Ketogenesis also takes place in the mitochondria of the hepatocytes and refers to the oxidation of FFA into ketone bodies to be oxidized by peripheral tissues (Laffel, 1999) as well as to serve as precursors for synthesis of fatty acids in the mammary gland (Drackley et al., 2001a). The FFA can also be re-esterified as triglycerides (**TG**), then packed and exported from the liver on very low-density lipoprotein (**VLD**) or used in

the TCA cycle after oxidation to acetyl CoA (Laffel, 1999, Newman et al., 2016). The uptake of FFA by the ruminant liver is influenced by the supply of FFA. Ruminant livers have a limited synthesis capacity to export TG; therefore, the TG accumulate in the hepatocyte. The correlation between FFA and BHB concentration is low (Figure 1.3) (Ospina et al., 2013, Leal-Yepes et al., 2014, McCarthy et al., 2015). Elevated postpartum blood concentrations of FFA and BHB are common during the NEB period. Concentration of BHB ≥ 1.2 mmol/L in blood is also known as hyperketonemia (**HYK**) and an increase in BHB concentration over this threshold is associated with negative outcomes (Ospina et al., 2010, Chapinal et al., 2011, McArt et al., 2012b).

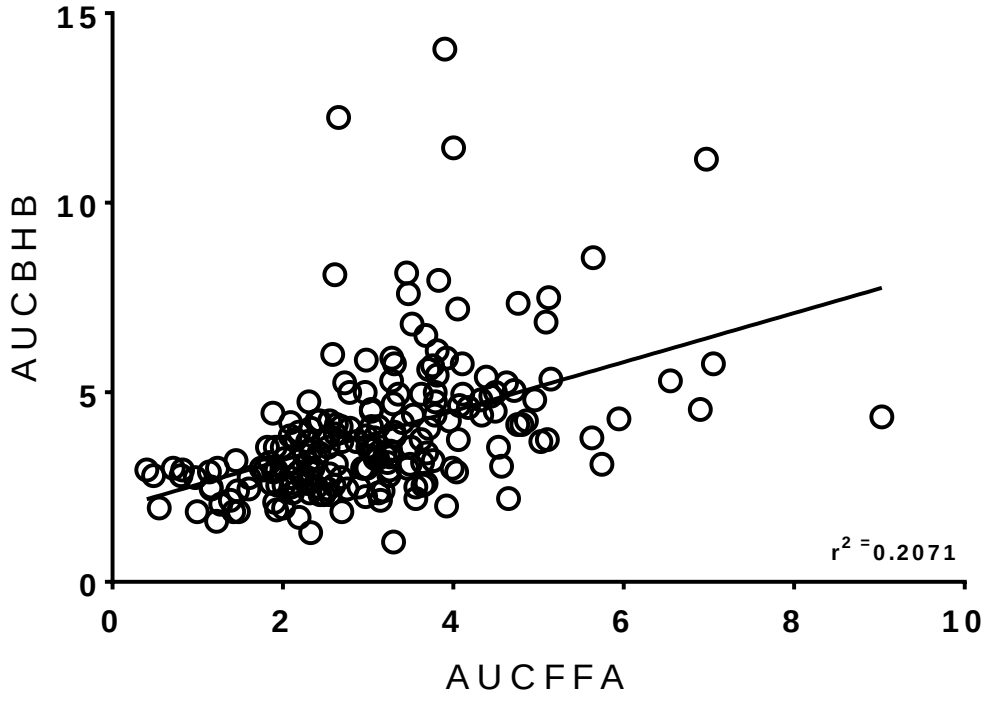

Figure 1.3. Linear regression, on the x-axis is area under the curve free fatty acids (FFA) and on the y-axis area under the curve β-hydroxybutyrate (BHB), adapted from McCarthy et al., (2015).

Approximately 40% of cows on a typical dairy farm will be hyperketonemic and the peak of HYK events occurs at 5 days in milk (**DIM**) (McArt et al., 2012a). Heritability of HYK and energy metabolites in blood in dairy cows has been reported as 0.02 (Gröhn et al., 1984), 0.06 (Uribe et al., 1995), 0.17 (van der Drift et al., 2012) and 0.39 (Van Dorp et al., 1998). Excessive NEB has been associated with increased incidence of displaced abomasum, metritis (LeBlanc et al., 2005, Caixeta et al., 2018), decreased milk yield, impaired immune function, and reduced fertility in dairy cows (Jorritsma et al., 2000, Leblanc, 2010, Contreras et al., 2012). Excessive NEB is costly and each case of HYK in the U.S. has been estimated to cost $289 USD (McArt et al., 2015) and might impact animal welfare.

Energy and protein metabolism

Undoubtedly, nutritional plans along with genetic selection programs have influenced the increased milk production in dairy cows over the past several decades. In order to increase the success of a cow between late pregnancy and early lactation, it is crucial to stimulate and increase gluconeogenesis in the liver. At the same time, a moderate break-down and posterior mobilization of body reserves is also beneficial to avoid the negative consequences generated by excessive lipolysis and proteolysis. During late pregnancy and early lactation, dietary energy and protein content can alter health status and production levels. Increasing the intake of energy stimulates protein synthesis in the mammary gland and nitrogen efficiency in the dairy cows (Broderick, 2003), indicating that there is a strong correlation among them.

Direct glucose absorption from the diet in ruminants is very low because the rumen microorganisms rapidly ferment non-structural carbohydrates. The result of the

ruminal fermentation process is production of volatile fatty acids. Propionate, a volatile fatty acid, after being absorbed through the rumen wall, can be used as substrates for gluconeogenesis in the liver. The nutrients are absorbed through the rumen wall or small intestine and transported to the liver via the portal vein. During the postprandial stage, glucose is stored as glycogen and synthesized into FFA or amino acids (**AA**).

Improving the efficiency (appropriate energy- protein balance) of dairy cows is a major area of study within the dairy industry not only in terms of profitability or welfare but also to reduce the emissions and pollutants produced by dairy farms (VandeHaar and St-Pierre, 2006). The metabolizable protein (**MP**) fraction is digested in different ways by ruminants: degraded to ammonia in the rumen; used for bacterial protein synthesis in the rumen; or, passes the rumen without degradation to be hydrolyzed in the abomasum then metabolized directly. The protein fraction is named based on the place where digestion occurs: rumen-degradable protein (**RDP**) and rumen- undegradable protein (**RUP**). The RDP fraction is utilized by ruminal microorganisms as well as the non-structural carbohydrates and is then used for protein synthesis or fermented to volatile fatty acids and ammonia. The RUP fraction is made up of microbial protein, ammonia, small peptides and AA that escape ruminal degradation. The small intestine absorbs small peptides and AA to be synthesized by the liver, mammary gland and peripheral tissues.

Feed is the most expensive input in dairy production systems. For that reason, MP should match metabolic needs for AA in order to maximize production and prevent health issues. Mobilization of protein from tissue is very important to supply AA for milk synthesis during the early postpartum period. A reduction in the diameter of

skeletal muscle was detected from calving until 6 weeks postpartum and the muscle loss is exacerbated by the EB of the cows (Mann et al., 2016).

The proteins and AA removed from body reserves are used mainly for gluconeogenesis in the liver or protein synthesis within cells and mammary gland. For that reason, the AA release from muscle may be involved in the regulation of EB. Supplementation of diets with rumen protected branched-chain amino acids during early postpartum may be a functional strategy to prevent adverse effects of excessive negative energy balance. Furthermore, small peptides produced and released by the muscle as well as AA, such as Leucine, can influence energy metabolism (Shimomura et al., 2006, Brandt and Pedersen, 2010). Muscle tissue in lactating dairy cows showed an upregulation of proteins involved in oxidation of FFA (Kuhla et al., 2011).

Hepatic lipidosis in dairy cows

Metabolism of FFA occurs mainly in the liver, adipose tissue and mammary gland but other organs can also oxidize FFA to spare glucose. Hepatic lipidosis occurs after excessive uptake of FFAs during early postpartum are then stored as TG in the hepatocyte. All cows accumulate TG in their liver postpartum and depending on the severity of the TG infiltration in the hepatocyte, hepatic lipidosis is categorized as normal (<1% wt. /wt.), mild (1-5% wt. /wt.), moderate (5-10% wt. /wt.), or severe (>10% wt. /wt.). Excessive hepatic lipidosis is toxic for the hepatocyte and impairs metabolic functions and stimulates cellular death (Jorritsma et al., 2001, Shibano and Kawamura, 2006).

Liver enzyme concentrations in plasma increase as an indicator of hepatocyte necrosis. Glycogen storage is affected, as well as the ability to pack, export, and oxidize

lipids from the hepatic tissue. Gluconeogenesis will be drastically reduced (Vernon, 2005). Peripheral tissues and organs are also affected due to low glucose concentration in blood and the toxic effects of increased concentration of FFA and ketone bodies in blood. Hepatic lipidosis causes detrimental effects on productive and reproductive performance in dairy cows (Veenhuizen et al., 1991, Jorritsma et al., 2000).

Propylene glycol (**PG**) has been used widely in dairy cows as treatment of HYK and hepatic lipidosis. Oral drench of PG is fermented in the rumen and absorbed as propionate and lactate from the rumen then utilized by the liver for gluconeogenesis. The PG oral administration also stimulates an increase in insulin secretion and decreased concentrations of FFA and BHB in blood (Grummer, 1993, Christensen et al., 1997, Nielsen and Ingvartsen, 2004). Total liver lipids and hepatic TG were reduced during early postpartum in cows treated with PG prepartum (Studer et al., 1993) and postpartum (Pickett et al., 2003).

BCAA metabolism

The liver also increases uptake of free AA for use in gluconeogenesis or as building blocks for protein. The branched-chain amino acids (**BCAA**; isoleucine, leucine and valine) are three of the known essential amino acids (**EAA**) in dairy cows. The BCAA are taken in excess of what is needed for milk synthesis by the mammary gland (Wohlt et al., 1977). Moreover, milk protein synthesis is not a direct response of MP absorbed by the small intestine (Lapierre et al., 2012).

Plasma free BCAA concentration in dairy cows drops several weeks prepartum, returning to normal concentration under regular conditions after two weeks postpartum (Kuhla et al., 2011, Zhou et al., 2016). Diets rich in BCAA have shown improvement

in glucose homeostasis and protein metabolism (Shimomura et al., 2006, Torres-Leal et al., 2011, Lynch and Adams, 2014) via the mammalian target of rapamycin (mTORC) (Kimball and Jefferson, 2006, Lynch and Adams, 2014, Yoon, 2016). The initial reaction of BCAA metabolism does not occur in the liver, as most of the other AA, because a low hepatic activity of branched-chain aminotransferase (BCAT). The initial deamination of BCAA is reversible to form glutamate and the corresponding branched-chain keto acids (BCKAs) (Takumi et al., 2011). The second reaction of BCAA catabolism, involves the enzyme branched-chain α-keto acid dehydrogenase (BCKD), irreversible decarboxylation of the BCKA to the corresponding branched-chain acyl-CoA esters (Holecek, 2018).

Post ruminal or intra-venous infusion of BCAA has been shown to increase protein concentration in milk (Rulquin et al., 2006, Appuhamy et al., 2011). BCAA supplementation reduced hepatic TG accumulation in monogastrics fed high-fat diets and improved liver function during hepatic lipidosis disease (Marchesini et al., 2005). BCAA supplementation upregulates the hepatic sterol regulatory element-binding protein/ liver x receptor pathway and are key factors in cholesterol and FFA metabolism. Also, BCAA supplementation may increase oxidation of FFA via peroxisome proliferator-activated receptor α (Arakawa et al., 2011, Bai et al., 2015).

In the following chapters, I present the results of two major studies aimed at: i) identification of genetic regions associated with different concentrations of FFA and BHB in plasma/serum in early postpartum Holstein cows, and ii) BCAA supplementation alone or in combination with oral administration of PG during early lactation in dairy cows. In the latter study, we hypothesized that BCAA supplementation

would have a beneficial role in milk yield and protein synthesis in the mammary gland as well as altering energy and protein blood metabolites associated with catabolic processes.

The identification of genomic regions associated with the development of hyperketonemia will improve our ability to identify the most susceptible animals to this metabolic diseases. Diets supplemented with rumen protected BCAA and glucose precursors during early postpartum may be a functional strategy to prevent adverse effects of excessive NEB. Use of these studies and future applied research will be fundamental to ensure the sustainable production in the dairy industry worldwide.

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CHAPTER 2

GENETICS OF HYPERKETONEMIA IN HOLSTEIN DAIRY COWS

Abstract

The objective of our study was to identify genomic regions associated with varying concentrations of non-esterified fatty acid (NEFA), β-hydroxybutyrate (BHB) and the development of hyperketonemia (HYK; blood BHB ≥1.2 mmol/L) in longitudinally sampled Holstein dairy cows. Our hypothesis was that genetic variations are associated with the capability to use NEFA efficiently, whereas other cows develop HYK. Our study population consisted of 147 multiparous cows intensively characterized with serial NEFA and BHB concentrations. Blood samples were collected 3 times per week and tested for NEFA and BHB concentrations. To identify individuals with contrasting combinations in BHB and NEFA concentrations, phenotypes were established using incremental area under the curve (AUC) and categorized as follows: group 1) high NEFA and high BHB (n = 10); group 2) low NEFA and high BHB (n = 11); group 3) low NEFA and low BHB (n = 69); and group 4) high NEFA and low BHB (n = 57). These categories allow us to distinguish cows based on their ability to mobilize, metabolize and oxidase NEFA and BHB. Holstein cows were genotyped on the Illumina Bovine High-density (777K) beadchip. Genome-wide association studies using mixed linear models with the least related animals (n=128) were performed to establish a genetic association with HYK, BHB-AUC, NEFA-AUC, and the comparisons of the 4 AUC phenotypic groups using Golden Helix software. Single-nucleotide polymorphisms with a false discovery rate $<1.81 \times 10^{-06}$ were used to identify candidate

genes. Nine single-nucleotide polymorphisms were associated with high concentrations of BHB and further investigated. Candidate genes including *LIPC* on chromosome 10, *HSD17B10* and *HTR2C* on chromosome X, and *ABCA1* and *ABCA2* on chromosome 8 were identified. These results might help identify susceptible animals thus improving genetic selection criteria to decrease the incidence of HYK.

Introduction

The ability of a cow to deal with extensive physiological changes during the late pregnancy and early postpartum period influences the entire lactation in terms of milk yield, quality, and health status (Drackley, 1999). Strong genetic selection of dairy cows, driven by the ability to achieve high milk production, particularly in early lactation, has increased the early postpartum gap between energy consumed and energy required (Veerkamp et al., 2003); however, some cows can overcome this crucial phase of metabolic adjustments when the energy demand is doubled immediately after calving (Drackley et al., 2001) without negative sequela for the animal's production or health.

During this period of negative energy balance (**NEB**), cows respond by mobilizing lipid and protein from tissue reserves in order to compensate for the reduced intake of nutrients (Drackley, 1999) and these reserves are used to support lactation and vital functions (Bauman and Currie, 1980). Fat reserves are released into the blood stream as non-esterified fatty acids (**NEFA**) that can be extracted and metabolized by several body tissues such as skeletal muscle, liver and kidney. Some cows adapt very well to NEB; however, other cows do not, resulting in excessive ketone body synthesis. Dairy cattle produce three different ketone bodies: acetoacetate, acetone and β-hydroxybutyrate (**BHB**). The BHB concentration in blood has been widely used to diagnose hyperketonemia (**HYK**) in dairy cattle (Iwersen et al., 2009). A low correlation between NEFA and BHB concentrations during the transition period was reported previously in a cross-sectional (Ospina et al., 2013) as well as longitudinal studies (Leal-Yepes et al., 2014, McCarthy et al., 2015). This shows that some cows seem to effectively use NEFA in the adaptation to lactation while having low BHB

concentrations, whereas other cows exhibit excessive ketone body synthesis.

Hyperketonemia is considered one of the most complex diseases in dairy cattle because there are many factors involved in its development such as advanced parity, increased body condition score before calving (McArt et al., 2013), nutrition during the dry period (Dann et al., 2006), over-crowded pens (Oetzel, 2007), and environment (Tveit et al., 1992). Up to 40% of cows on a typical dairy farm will be hyperketonemic, and the peak of HYK incidence occurs at 5 days in milk (**DIM**) (McArt et al., 2012). Hyperketonemia may increase metabolic diseases and reduce milk production (Grummer, 1993, Duffield et al., 2009, Ospina et al., 2010b). The cost per individual cow case of HYK in the U.S. has been estimated to be $289 accounting all the direct and indirect costs associated with the disorder (McArt et al., 2015). Heritability of HYK or concentrations of energy metabolites in blood in dairy cows has been reported to range from 0.02 to 0.39 (Gröhn et al., 1984, van Dorp et al., 1998, van der Drift et al., 2012, Weigel et al., 2017). The difference between the heritability reported in previous studies may be due to differences in the characterization of HYK and degree of NEB during early lactation in dairy cows.

A genome-wide association study (**GWAS**) allows us to analyze in detail the relationship between genotypic and phenotypic data, thereby associating single-nucleotide polymorphism **(SNP)** allelic frequencies to disease susceptibility (Laurie et al., 2010). A genetic analysis can identify the heritability of quantitative traits that are risk factors for the disease (Hirschhorn and Daly, 2005). We hypothesized that there are genetic variations that are associated with the capability to use NEFA efficiently during early lactation whereas other cows develop HYK. Our intensively characterized data set

allowed us to isolate genetic differences of cows within similar environmental and management conditions. Therefore, the objective of our study was to identify genomic regions associated with the development of HYK (BHB ≥1.2 mmol/L) based on serial measures reflecting different concentrations of NEFA and BHB over time in early postpartum Holstein cows.

Materials and methods

Sampling procedure

All procedures were approved by the Cornell University Institutional Animal Care and Use Committee (protocols no. 2008-0099 and 2011–0016). Our study population consisted of 147 Holstein dairy cows from two trials. Both trials evaluated multiparous cows having serial measurements of serum/plasma NEFA and blood BHB concentration from calving until 16 DIM 3 times per wk. Blood samples were collected from coccygeal vessels. Serum was tested for NEFA concentration (HR Series NEFA-HR (2); Wako Life Sciences, Mountain View, CA) and BHB using a cow side test (Precision Xtra meter, Abbott Diabetes Care Inc, Alameda, CA, USA). This cohort of cows were not treated for hyperketonemia during either of the trials. Detailed information about the first study evaluating 63 cows was previously reported (McArt et al., 2011). An extra blood sample (7 mL) was harvested once using evacuated glass tubes (Beckton Dickinson Vacutainer System, Franklin Lakes, NJ) with K_3 EDTA and a 20-gauge x 2.54 cm blood collection needle for subsequent DNA extraction. After collection, blood tubes were gently inverted 5 times to homogenize blood with K_3 EDTA and immediately placed on ice to prevent DNA degradation. After that, blood samples were then stored at -20°C until DNA extraction and subsequent genotyping. The second

trial similarly evaluated 84 cows (Mann et al., 2015). DNA was extracted from muscle biopsies which were performed for all cows within the study (Mann et al., 2016). The muscle biopsies were placed on liquid nitrogen and stored at -80°C until DNA extraction and subsequent genotyping.

DNA extraction and genotyping

Whole blood or muscle tissue were submitted for DNA extraction and genotyping to GeneSeek laboratories (Lincoln, NE, USA). The DNA extraction was performed with Omega Mag Bind Tissue kit for DNA extraction following manufacturer's instructions (OMEGA bio-tek, Norcross, Georgia, USA). Whole-genome genotypes of 777,962 SNPs were generated using the Illumina Bovine High-density beadchip (Rincon et al., 2011). Genotype data were filtered using Golden Helix SNP & Variation Suite (SVS) 8.3.4 software (Golden Helix, Bozeman, MT, USA). Sample quality control was performed, and 19 samples (12.9%) with a call rate <0.90 were excluded. Genetic quality control was performed excluding SNPs with a call rate <0.90, minor allele frequency (**MAF**) <0.05 or if the number of alleles was ≥2. A total of 521,929 SNPs remained for analysis after quality control filtering.

Quantile–quantile (**Q–Q**) plots have been broadly used to graphically detect problems in a GWAS by representing the deviation of the observed *P*-values from the expected *P*-values. In order to check our genetic association models, we plotted the observed –log 10 *P*-values on the y-axis against the expected –log 10 *P*-values on the x-axis.

Phenotypic classification

There is a lack of information regarding the genetic factors predisposing cows to HYK due to the complexity of the disease. The aforementioned trials from which these biological samples derived, allowed for a unique opportunity to investigate these genetic parameters using 6 serial measurements of BHB and NEFA concentration. Multiple phenotypic classifications were established to identify variation in the genetic regulation of HYK. First, HYK was defined as concentration in blood of BHB (≥1.2 mmol/L) at a minimum of one time point from calving until 16 DIM, corresponding to previous characterization of HYK (Duffield et al., 2009, Ospina et al., 2010a).

A second, broader approach was also taken by calculating the incremental area under the curve (**AUC**) using the 6 concentrations of serum/plasma NEFA or blood BHB from the longitudinally sampled cows from calving until 16 DIM. The trapezoidal rule was used to estimate AUC by summing the area of all the trapezoids formed between two time points (Chiou, 1978) with the statistical software package SAS 9.4 (SAS Institute Inc., Cary, NC). The AUC allowed us to compile the serial measurements from each individual into a single continuous variable and preserve the variability within the dataset as opposed to using either a single measure of the given trait e.g. HYK, or cow average for the trait. We expect that phenotypic misclassification in our study population would be lower using this approach.

All modern dairy cows will face NEB during early lactation and some will mobilize body reserves as NEFA to a greater extent whereas others to a lesser extent. Therefore, ketogenesis magnitude will differ among cows as reflected by circulating BHB. These parameters were used to categorize cows into groups that contrast their ability to mobilize as well as metabolize energy reserves. The resulting AUC were used

to group cows to identify individuals with the most variation during the first 16 DIM. For BHB area under the curve (**BHB-AUC**), a high concentration was defined at values >7.2 mmol/L; this threshold was generated by computing the AUC of 6 single measurements of BHB >1.2 mmol/L (McArt et al., 2011). For NEFA area under the curve (**NEFA-AUC**), a high concentration was considered at values >4.2 µEq/L; this value was set by calculating the AUC of 6 single measurements of NEFA of >0.7 µEq/L (Ospina et al., 2013). Therefore, in our study, all cows were then classified into 4 different phenotype groups based on their NEFA-AUC and BHB-AUC as follows: group 1) high NEFA and high BHB (n = 10); group 2) low NEFA and high BHB (n = 11); group 3) low NEFA and low BHB (n = 69); and group 4) high NEFA and low BHB (n = 57). The variables BHB-AUC and NEFA-AUC were not normally distributed, therefore their values are given as median and range. The variables BHB-AUC and NEFA-AUC were analyzed using the non-parametric Kruskal-Wallis test with PROC NPAR1WAY (SAS 9.4, SAS Institute, Cary, NC). Chi-square tests using PROC FREQ (SAS 9.4) were performed to identify differences among the variables farm, parity and HYK when phenotype groups were used as the response variable.

Genome-Wide Association Study

The degree of relatedness between pairs of cows in this study was computed to identify highly related animals using genomic identity-by-descent (**IBD**) estimations in SVS 8.3.4 software (Golden Helix, Bozeman, MT). The IBD estimates the likelihood of specific alleles being inherited from a common ancestor when comparing two individual samples. IBD estimates allowed for the identification and removal, if

necessary, of highly related animals in lieu of pedigree information to minimize the risk of false positives results (Laurie et al., 2010) (Figure 2.1).

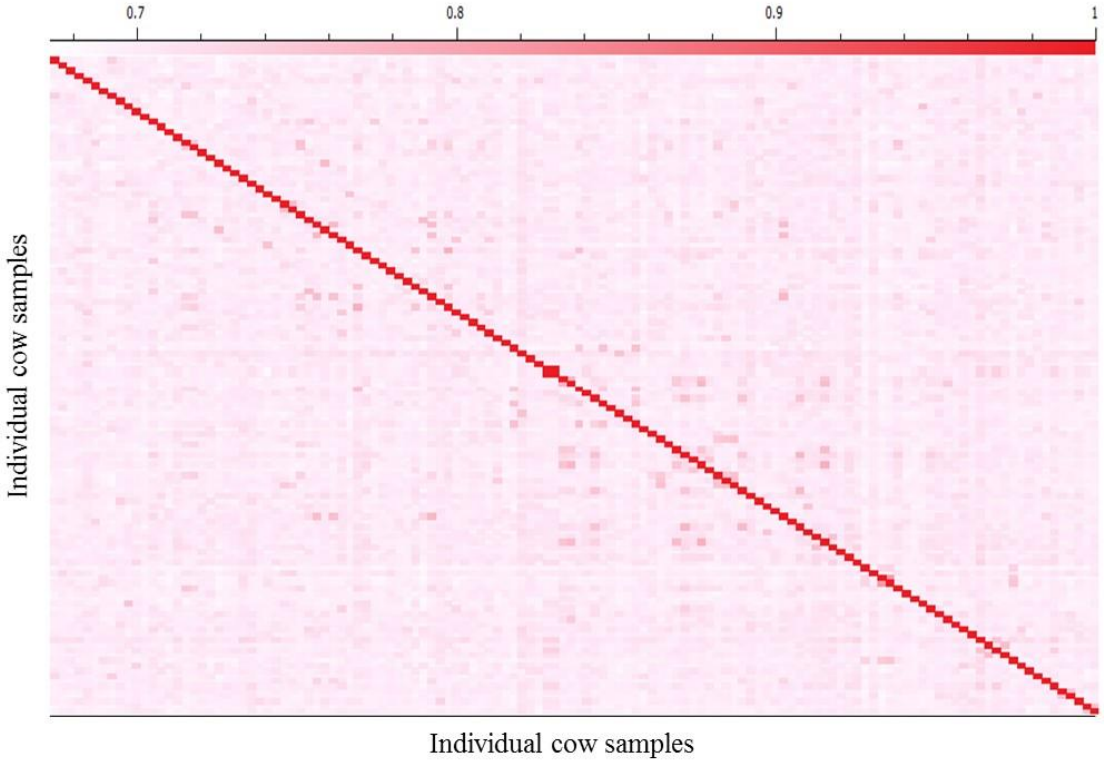

Figure 2.1. A heat map depiction of the genomic kinship matrix showing the relatedness between each cow sample and general population structure. This matrix is calculated using the identity-by-descent (IBD) procedure from SVS (Golden Helix) Software. The X- and Y- axes correspond to the 128 least related cow individuals. Highly related cows were removed to minimize the risk of false positive results.

Genome-wide association studies were performed to establish associations between low frequency SNP variants and the development of HYK or different levels of BHB and NEFA in early postpartum Holstein dairy cows. The Efficient Mixed Model Association eXpedited (**EMMAX**) algorithm is a mixed linear model embedded in Golden Helix software which corrects for population stratification and relatedness (Kang et al., 2010) and was used to perform the GWAS analysis. This model was the most suitable because the population structure is taken into account by including the

kinship matrix previously generated with the IBD procedure. The kinship matrix was included as the variance-covariance structure of the random effect for the individuals (Zhang et al., 2010). The single-locus mixed model GWAS EMMAX uses the following general equation, $y = X\beta + u + e$, where **y** is an $\boldsymbol{n}\times\mathbf{1}$ vector of observed phenotypes, X is an $\boldsymbol{n}\times\boldsymbol{q}$ matrix of fixed effects including mean, SNPs and other confounding variables. Beta is a $q \times 1$ vector representing coefficient of the fixed effects. U is the random effect of the mixed model with $\mathrm{Var}(\mathbf{u}) = \sigma_g^2 K$, where *K* is the kinship matrix inferred from the genotype and e represents the error term and is the residual that cannot be explained by the variables in the model (Kang et al., 2008). HYK was evaluated as a categorical variable with individuals designated as case, equating HYK diagnosis, or control. Similarly, a pair-wise evaluation was conducted by comparing designated Groups having differential BHB-AUC and NEFA-AUC measures to one another. Thereby, Group 1 was independently compared to each remaining Group in separate GWAS. We note that the GWAS comparing Group 1 to Group 2 was not performed due to the extremely low number of individuals in both Groups (n= 11; n=10 respectively). Lastly, the measures of BHB-AUC and NEFA-AUC were analyzed independently as continuous variables in their respective GWAS. Parity, farm, milk production, and disease events (i.e. displaced abomasum, metritis, and retained placenta) were evaluated for inclusion in the model. Specific diet and dry matter intake were unavailable. Health events were excluded due to inconsistency in farm recording. Milk production was excluded due to an incomplete data set and subsequent unbalancing of the model. Parity and farm were retained as fixed effects in all GWAS. Multiple testing correction using false discovery rate (**FDR**) was performed to diminish the probability of Type I error.

The FDR was calculated using the formula FDR~ $1/k \sum_{i=1}^{K} \Pr(Hoi|Y)$, where K is equal to the number of SNPs used on the final examination (Benjamini and Hochberg, 1995, Gondro et al., 2008). Candidate genes were located by referencing 0.5Mbp up- and 0.5Mbp down-stream from the significantly associated SNPs passing multiple testing correction using the University of Maryland (**UMD**) 3.1 bovine genome assembly. Genes which showed biological plausibility in hyperketonemia were highlighted for discussion.

Pseudo heritability or narrow-sense heritability was calculated for HYK, BHB-AUC and NEFA-AUC using the formula $h2 = \sigma 2g/(\sigma 2g + \sigma 2e)$, were h^2 is the response heritability, σ^2_g is the genetic variance and σ^2_e variance caused by the environment. The GWAS analysis partitions the observed phenotypic variance into the additive genetic and non-genetic components. This estimation can be used to determine heritability, also known as pseudo-heritability (Zaitlen and Kraft, 2012, Korte and Farlow, 2013).

Haplotype analysis

Linkage disequilibrium (**LD**) was calculated using the expected maximization logarithm (**EM**) to derive r^2 estimates of pairwise LD using the SVS software. Linkage disequilibrium was computed to identify haplotype blocks and their potential association with the phenotypic variables. Haplotype analysis enhances the information obtained from the association test by incorporating the information from multiple markers among the same gene or genes with minimal historic recombination. The expectation maximization (**EM**) algorithm embedded in Golden Helix SVS software was used to compute haplotype frequencies. The EM algorithm is an iterative

technique that starts with arbitrary values (**Expectation step**), and these values are used to calculate the haplotype frequencies by maximum likelihood (**Maximization step**). This algorithm allows us to perform an automatic detection of LD block through the whole genome.

Haplotype Trend Regression (**HTR**) takes one or more blocks of genotypic markers and for each block of markers, estimates haplotypes and then regresses their by-sample haplotype probabilities against a dependent variable. Haplotype analysis is an important part of association testing as it can be sensitive to unmeasured variants which may be missed in a single SNP analysis (Stram, 2014). It can also provide an alternative marker panel consisting of a series of consecutive markers, therefore less sensitive to the effects of recombination on prediction accuracy for use in genomic selection.

Results

Twenty-nine percent of the individuals (n=42) were defined as HYK at a minimum of one time point from calving until 16 DIM in our data set. We confirmed that misclassification of HYK in our study population is lower using the serial measures as opposed to one opportunity afforded by cross sectional studies. This is evident in that the percentage of individuals defined as HYK at a single time point ranged from 7% (time point 1) to 15% (time point 3). Indeed, thirty-six percent of the HYK individuals (n=15 out of 42) only had a single measure of BHB concentration $\geq$1.2mmol/L out of the six time points and were therefore probable candidates for misclassification if this were a cross-sectional study. In contrast, twenty-six percent of the HYK individuals (n=11 out of 42) were likely to be diagnosed as HYK regardless of time point given that

they had four or more elevated measures of BHB (≥1.2mmol/L). By using the average BHB concentrations for individuals, only 12% of the study cohort (n=17) would have been diagnosed HYK. The variation in HYK designation when comparing single time points as given in a cross-sectional study or average BHB concentrations to our definition of HYK (min of 1 elevated BHB) showcases the relevance of using serial measures for phenotypic classification of hyperketonemia.

To further refine our phenotypic characterization of hyperketonemia, we calculated area under the curve for the serial BHB and NEFA concentrations. This allowed us to distinguish individuals who had a single elevated concentration of BHB or NEFA respectively from those who had two, three, four, five, or six episodes of elevated BHB or NEFA. It also incorporated concentration variation into the calculated AUC variable thereby distinguishing individuals demonstrating particularly high concentrations (i.e. BHB 3.9mmol/L) from those with lower concentrations including the HYK defined minimum threshold of 1.2mmol/L. The NEFA-AUC ranged from 0.40 to 9.03 µEq/L over 16 d with a median of 3.92 µEq/L, and the BHB-AUC ranged from 1.60 to 14.25 mmol/L over 16 d with a median of 3.65 mmol/L (Figure 2.2). The distribution of parity, HYK, and farm was also analyzed, and total counts are shown in Table 2.1 based on the Group designation.

The Q-Q plots from the different mixed linear model using EMMAX are shown in Figure 2.3 and Figure 2.4. These plots showed most of the observed –log 10 (*P*-Value) following a uniform distribution, indicating that our genetic quality control was appropriate. Moreover, Q-Q plots are showing few uncorrected $\log_{10}$ transformed P-values located in the tail of the plots with a significant deviation of the expected

uncorrected $\log_{10}$ transformed P-values, in agreement with the results obtained from the different Manhattan plots.

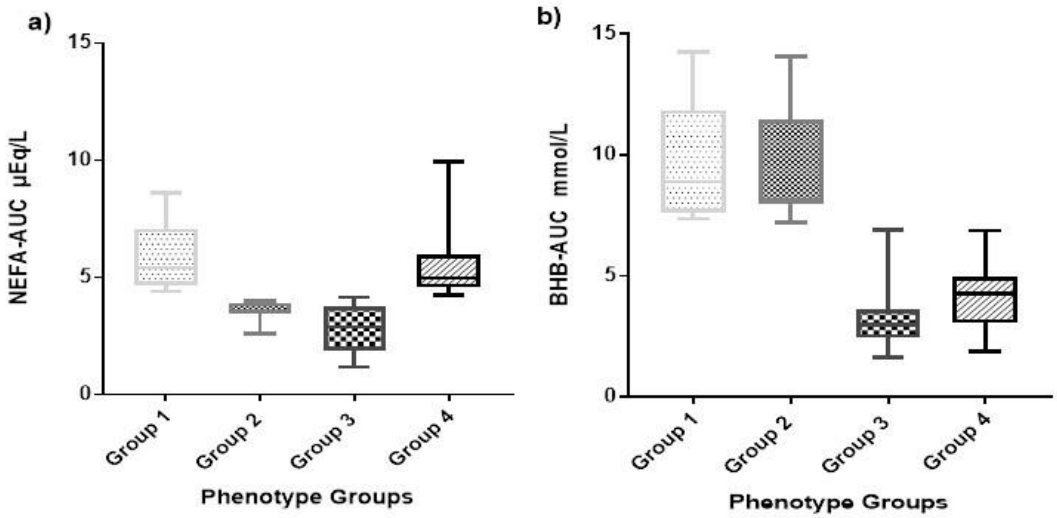

Figure 2.2. Non-esterified fatty acids area under the curve (NEFA-AUC) and BHB area under the curve (BHB-AUC) box and whisker plot. a) The box and whisker plot showing the distribution of NEFA-AUC µEq/L during the first 16 DIM by phenotype group; b) The box and whisker plot showing the distribution of BHB-AUC mmol/L during the first 16 DIM by phenotype group. The phenotypes groups are: 1 (high NEFA-AUC and high BHB-AUC), 2 (Low NEFA-AUC and high BHB-AUC), 3 (Low NEFA-AUC and low BHB-AUC), and 4 (High NEFA-AUC and low BHB-AUC).

Table 2.1. Descriptive statistics of the study population. Results are presented as total counts or range of values and median value.

Groups[1]		**1**	**2**	**3**	**4**	**Overall Count**	***P*-value**
Total per group		10	11	69	57	147	
Parity number [2]	2	1	4	18	12	35	0.003
	3	1	3	37	25	66	
	≥4	8	4	14	20	46	
Hyperketonemic[3]		10	11	5	16	42	<0.0001
BHB-AUC[4]		8.85 (7.3-14.2)	8.1 (7.2-14)	2.95 (1.6-6.9)	4.25 (1.9-6.9)	3.65 (1.6-14.2)	<0.0001
NEFA-AUC[5]		5.41 (4.4-8.6)	3.84 (2.6-4)	2.89 (1.16-4.1)	5 (4.2-9.9)	4 (1.16-9.9)	<.00001
Farm[6]	1	4	4	11	11	30	0.05
	2	0	4	12	17	33	
	3	6	3	46	29	84	

1 Group 1) High non-esterified fatty acids (NEFA) and high BHB, Group 2) Low NEFA and high BHB , Group 3) Low NEFA and low BHB , and Group 4) High NEFA and low BHB;

2 Difference in parity between the groups was calculated with Chi-Square;

3 Hyperketonemia was the number of cows in each group with a single measurement of BHB ≥1.2 mmol/L during the first 16 DIM and the difference was calculated using Chi-square;

4 BHB-AUC are shown as the range of values and median value. The difference in BHB-AUC was calculated with non-parametric Kruskal-Wallis test.

5 BHB-AUC are shown as the range of values and median value. The difference in BHB-AUC was calculated with non-parametric Kruskal-Wallis test.

6 Farm is expressed as counts and was analyzed with chi-square.

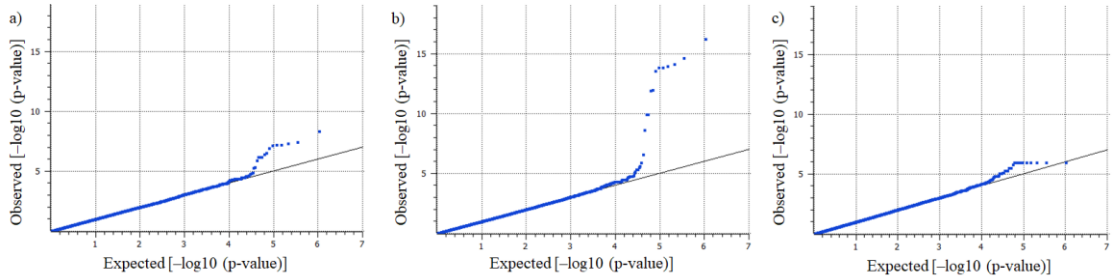

Figure 2.3. Quantile-Quantile plot (QQ-plot) of –log 10 (*P*-Value) from mixed linear models with parity and farm as fixed effects: a) Hyperketonemia; b) BHB-AUC and c) NEFA-AUC. The expected –log 10 (*P*-Value) are presented on the x-axis and the observed P-values are on the y-axis.

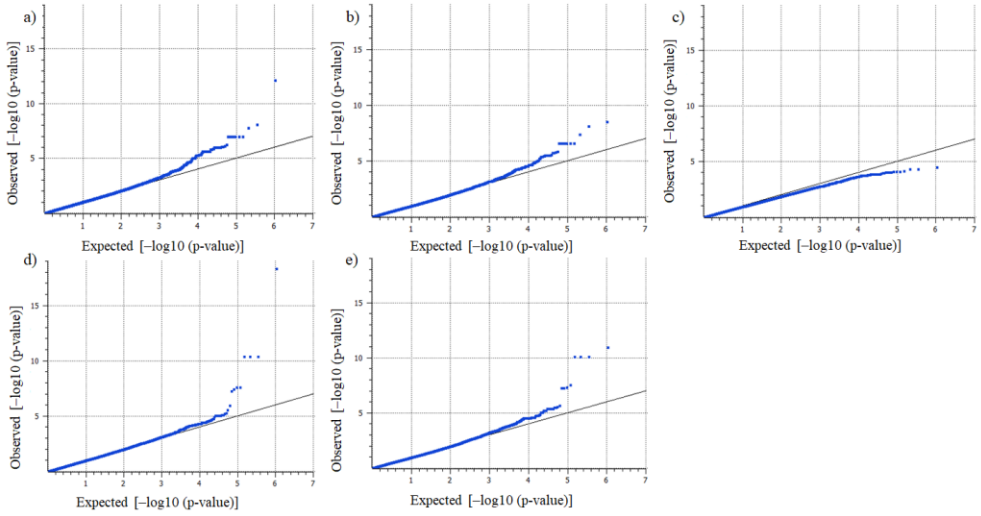

Figure 2.4. Quantile-Quantile plot (QQ-plot) of –log 10 (*P*-Value) from mixed linear models with parity and farm as fixed effects: a) phenotype group 1 (high NEFA-AUC and high BHB-AUC) Vs phenotype group 3 (Low NEFA-AUC and low BHB-AUC); b) phenotype group 2 (Low NEFA-AUC and high BHB-AUC) Vs phenotype group 3 (Low NEFA-AUC and low BHB-AUC); c) phenotype group 3 (Low NEFA-AUC and low BHB-AUC) Vs phenotype group 4 (High NEFA-AUC and low BHB-AUC); d) phenotype group 1 (high NEFA-AUC and high BHB-AUC) Vs phenotype group 4 (High NEFA-AUC and low BHB-AUC); and e) phenotype group 2 (Low NEFA-AUC and high BHB-AUC) vs phenotype group 4 (High NEFA-AUC and low BHB-AUC). The expected –log 10 (*P*-Value) are presented on the x-axis and the observed P-values are on the y-axis.

We performed 8 different GWAS using HYK as a dichotomous phenotype, BHB-AUC as a continuous phenotype, NEFA-AUC as a continuous phenotype, and the 5 possible pair-wise combinations of the 4 AUC phenotype groups. Figure 2.5 shows the Manhattan plots of HYK, BHB-AUC, and NEFA-AUC using the uncorrected $\log_{10}$ transformed P-values. Results for HYK and BHB-AUC are very similar which is to be expected with HYK diagnosis being dependent upon BHB concentrations. That being said, BHB-AUC results show consistently higher degrees of association for the majority of the significant SNPs which is likely reflective of using the area under the curve approach encompassing a greater degree of the variation from the 6 serial measures. This can be seen with markers on chromosomes 4, 5, 8, 10, 16, and X. The NEFA-AUC GWAS did not provide any significant associations after multiple testing correction. The GWAS reflecting the pairwise comparisons of the categorical groups is shown in Figure 2.6. Similar to HYK and BHB-AUC results, the categorical grouping gave the most promising results when comparing high BHB concentration groups (Groups 1 & 2) to low BHB concentration groups (Groups 3 & 4) (Fig 2.6.a.b.d.e). No significant associations were identified when only comparing variation in NEFA concentrations (Fig 2.6.c). Despite the similar general outcomes reflecting BHB concentrations, this categorical approach shows genomic variation potentially related to NEFA mobilization as seen by markers on chromosome 3 in Figure 2.6.a as opposed to Figure 2.6.b. These GWAS compare Groups 1 or 2, both having high BHB-AUC partnered with either high NEFA-AUC or low NEFA-AUC respectively, to Group 3 which reflects animals with both low BHB and NEFA-AUC.

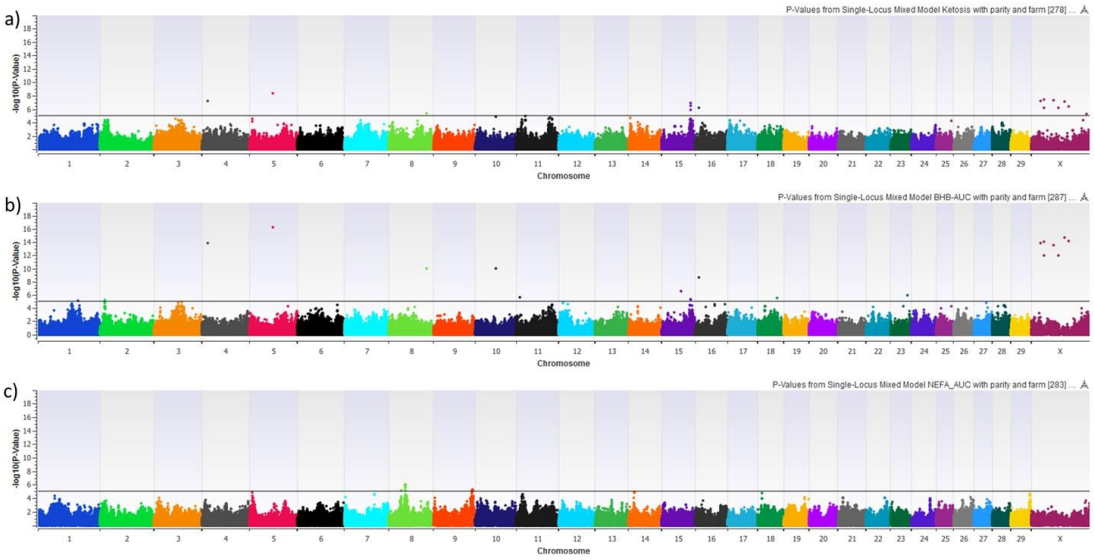

Figure 2.5. Manhattan plot showing the graphic representation of –log 10 (*P*-Value) from mixed linear models with parity and farm as fixed effects for a) Hyperketonemia; b) BHB-AUC and c) NEFA-AUC. SNPs above the horizontal black lines achieved a false-discovery rate corrected *P*-value of <0.05 and were explored for biological significance.

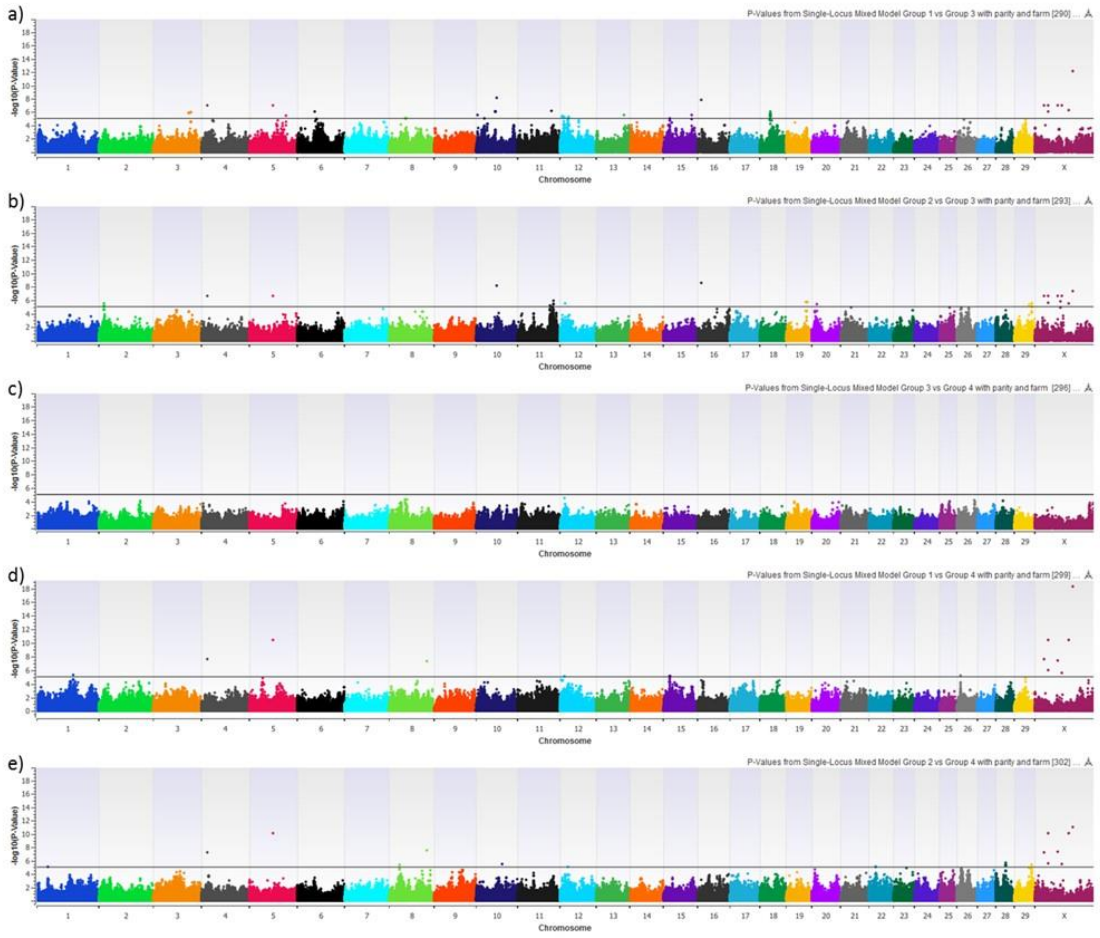

Figure 2.6. Manhattan plot showing the graphic representation of –log 10 (*P*-Value) from each different mixed linear model. Specifically, this plot corresponds to the mixed linear model with parity and farm as fixed effects: a) phenotype group 1 (high NEFA-AUC and high BHB-AUC) Vs phenotype group 3 (Low NEFA-AUC and low BHB-AUC); b) phenotype group 2 (Low NEFA-AUC and high BHB-AUC) Vs phenotype group 3 (Low NEFA-AUC and low BHB-AUC); c) phenotype group 3 (Low NEFA-AUC and low BHB-AUC) Vs phenotype group 4 (High NEFA-AUC and low BHB-AUC); d) phenotype group 1 (high NEFA-AUC and high BHB-AUC) Vs phenotype group 4 (High NEFA-AUC and low BHB-AUC); and e) phenotype group 2 (Low NEFA-AUC and high BHB-AUC) vs phenotype group 4 (High NEFA-AUC and low BHB-AUC). SNPs above the horizontal black lines achieved a false-discovery rate corrected *P*-value of <0.05 and were explored for biological significance.

Nine SNPs (Table 2.2) passing multiple testing correction (FDR≤0.05) were explored for candidate genes with a biological relationship with changes in NEFA, BHB or the development of HYK during the early lactation period. The region on chromosome 5 as well as four regions on the X chromosome lacked bovine annotated genes or did not provide candidate genes of biological relevance to hyperketonemia in other reference genomes. Hydroxysteroid (17-beta) dehydrogenase 10 *(HSD17B10)*, ATP-binding cassette transporter 1 *(ABCA1)*, and hepatic lipase *(LIPC)* genes were identified on chromosomes X, 8, and 10 respectively using the bovine reference genome. 5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled *(HTR2C)* and ATP-binding cassette transporter 2 *(ABCA2)* genes were identified on chromosomes X and 8 respectively using gene homology to human and mouse assemblies.

Table 2.2. Genome-wide association study results identifying associated SNPs and candidate genes from the 128 least related Holstein cows.

Chr	SNP location [1]	Gene Name	Gene Start[2]	Gene End[2]	Distance to the SNP(bp)[3]	Best P-value[4]	False Discovery Rate P-value	Phenotypes associated by GWAS[5]
5	60045785	*KIAA0748*	60276249	60300244	254459	5.98x10-17	3.12x10-11	HYK, BHB-AUC, 1 vs 3, 1vs 4, 2 vs 3 and 2 vs 4
		AMOTL2	60276249	60300244	254459			
		TESPA1	60265872	60301998	256213			
		NEUROD4	60223354	60234099	177569			
8	95966003	*ABCA1, ABCA2*	96270791	96408375	304788	1.14x10-10	5.96x10-06	HYK, BHB-AUC, 1vs 3, 1 vs 4 and 2 vs 4
		OR13F1	95761537	95762465	42726			
		NIPSNAP3A	96239004	96252150	273001			
10	51462618	*LIPC*	51758867	51921040	296249	1.14x10-10	5.45x10-06	HYK, BHB-AUC, 2 vs 3 and 2 vs 4
		MYO1E	51020004	51240914	221704			
		CCNB2	51247755	51272733	189885			
		MINDY2	51500299	51570991	37681			
		RNF111	51288677	51380159	82459			
		ADAM10	51598073	51739157	135455			
		CLNS1A	51841987	51843484	379369			
X	32696913	*MAGEA*	32428998	32719060	22147	3.67x10-15	6.39x10-10	HYK, BHB-AUC, 1 vs 3, 1 vs 4, 2 vs 3 and 2 vs 4
		IDS	32302897	32324344	372569			
		CXorf40A	32345274	32348925	347988			
		TMEM185A	32922144	32957365	225231			
X	32726008	*TMEM185A*	32922268	32964027	196260	2.27x10-13	1.48x10-08	

		IDS	32302897	32324344	401664			HYK, BHB-AUC, 1 vs 3, 1 vs 4, 2 vs 3 and 2 vs 4
		MAGEA	32685989	32687155	38853			
		HSFX3	32590840	32592421	133587			
		CXorf40A	35613768	35614939	2888931			
X	57467501	*BEX3*	57269171	57270812	196689	4.35x10-15	5.68x10-10	HYK, BHB-AUC, 1 vs 3, 1 vs 4, 2 vs 3 and 2 vs 4
		CXorf57	56957121	57062226	405275			
		TCEAL9	57244995	57246736	220765			
		PLP1	57864748	57881720	397247			
		RAB9B	57864581	57926698	397080			
		MORF4L2	57744023	57754954	276522			
		GLRA4	57785197	57798407	317696			
X	68194066	*HTR2C*	67986710	68083180	110886	4.42x10-15	3.30x10-10	HYK, BHB-AUC, 1 vs 3, 1 vs 4, 2 vs 3 and 2 vs 4
		LHFPL1	68428002	68491176	297110			
		SNORA35	67792018	67792065	402001			
		AMOT	68598671	68657930	404605			
		RTL4	68212448	68289758	18382			
		RBMX2	68131773	68142135	51931			
X	85371223	*SLC7A3*	85010185	85015654	361038	2.70x10-12	1.57x10-07	HYK, BHB-AUC, 2 vs 3 and 2 vs 4
		DLG3	85245062	85298606	72617			
		GDPD2	85309754	85319162	52061			
		KIF4A	85321378	85449490	0			
		P2RY4	85481339	85482436	110116			
		AWAT1	85499886	85508546	128663			
		DGAT2L6	85556422	85577736	185199			
		IGBP1	85588914	85635721	217691			
		EDA	85708003	86099973	336780			

		IL2RG	84816145	84819841	551382			
		IKZF5	85444282	85447321	73059			
X	95872578	*HSD17B10*	96267144	96269467	396889	4.03x10-16	2.10x10-10	HYK, BHB-AUC, 1 vs 3, 1vs 4 and 2 vs 4
		MAGED4B	95546166	95553643	318935			
		GPR173	95933520	95953706	60942			
		KDM5C	96041773	96072571	169195			
		SMC1A	96218220	96252806	345642			
		RIBC1	96253141	96266987	380563			
		HUWE1	96362881	96520246	490303			

[1] SNP location: exact location of the SNP within the chromosome referencing the University of Maryland (**UMD**) 3.1 bovine genome assembly;

[2]Gene start and Gene end :The coordinates of beginning and end for genes located on the region of influence of associated SNPs; the region of influence of each SNP was defined as 1 Mega base pair up and downstream on the UMD 3.1 bovine genome assembly;

[3]Distance to the SNP (bp): Distance in base pairs between the SNP and the candidate gene;

[4] Best p-value: indicates the lowest p-value when the SNP was significant in multiple GWAS; only SNPs with a corrected FDR $\leq$ 0.05 were analyzed to diminish the probability of Type I error;

[5] Shows all different explanatory variables where the SNP was significant with a corrected FDR $\leq$ 0.05; GWAS: Hyperketonemia (HYK/dichotomous), BHB-AUC (continuous response), Group 1 vs Group 2 (dichotomous), Group 1 vs Group 3 (dichotomous), Group 1 vs Group 4 (dichotomous), Group 2 vs Group 3 (dichotomous), Group 2 vs Group 4 (dichotomous) and Group 3 vs Group 4 (dichotomous).

In our study, the pseudo-heritability of HYK was 0.16±0.56; BHB-AUC 0.82±0.44; and NEFA-AUC 0.02±0.14. Standard errors for pseudo-heritability are likely magnified due to the small sample size yet reflect similar estimates of previous studies. Haplotype analysis showed no significant association after testing for haplotypes frequencies with HTR (*P*-value=0.65). In all, significant associations were identified on four chromosomes and highlighted five biologically plausible candidate genes. The small sample size likely limited potential findings while the use of the serial NEFA and BHB measures mitigated this issue by improving the accuracy of the phenotypic characterization.

Discussion

Our study aimed to identify genomic regions associated with different concentrations of NEFA, BHB, and the development of HYK in early postpartum multiparous Holstein cows. Hyperketonemia is a complex disorder that has many potential risk factors including genetic factors (Gondro et al., 2013). Accuracy of the diagnosis of HYK and the intricacies of BHB and NEFA concentration variation play a crucial role in identifying genomic regions associated with this disease. The in-depth phenotyping with the longitudinal sampling during the first 16 DIM allowed for a more accurate assessment of HYK as opposed to single measurement derived from a cross-sectional study design and mitigated the effect of the small sample size.

Multiple GWAS assessing 521,929 SNPs from 128 least-related Holstein cows identified 9 SNPs highlighting 5 candidate genes with biological relevance in the development of HYK or with high AUC for BHB and NEFA concentrations within the associated region.

Candidate genes

Hydroxysteroid (17-beta) dehydrogenase 10 (HSD17B10). This gene is located on chromosome X from 96,267,144 - 96,269,467 base pairs (**bp**) (RefSeq: NM_174334.3) and is well conserved in all vertebrates (Mindnich et al., 2004). In 4 out of 8 GWAS, ***HSD17B10*** emerged as a candidate gene: Hyperketonemia (dichotomous), BHB-AUC (continuous), group 2 vs group 3 (dichotomous), and group 2 vs group 4 (dichotomous). Yang et al. (2009) reported that *HSD17B10* gene encodes for a mitochondrial multifunctional enzyme, which catalyzes the oxidation of steroid modulators of gamma aminobutyric acid type A receptors and steroid hormones. It also has short-chain 3-hydroxy-2-methylacyl-CoA dehydrogenase activity, an essential step in the degradation of isoleucine. Isoleucine is an essential branched chain amino acid (EAA) that plays a pivotal role in protein and energy metabolism (Kuhla et al., 2011) with particular importance due to the contribution to milk protein synthesis (Mackle et al., 1999). Mutations in *HSD17B10* have been reported in humans and caused a complete loss of a mitochondrial multifunctional enzyme which were biochemically diagnosed with an elevated concentration of metabolites from isoleucine breakdown (Zschocke, 2012). Given *HSD17B10's* role in energy metabolism and protein synthesis, we hypothesize that this gene's activity and efficiency may play a role in hyperketonemia.

5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled (HTR2C). *HTR2C* gene is located on chromosome X from 67,986,710 - 68,083,180 bp (RefSeq: AC_000187.1). In 6 out of the 8 performed GWAS, ***HTR2C*** was identified as a candidate gene: HYK (dichotomous), BHB-AUC (continuous), group 1 vs group 3

(dichotomous), group 1 vs group 4 (dichotomous), group 2 vs group 3 (dichotomous), and group 2 vs group 4 (dichotomous). The hypothalamus consolidates all the processes related with energy homeostasis. The ability of transition cows to gain energy homeostasis is vital as they manage NEB and move towards neutral and positive energy balance which relates to the relevant events and key time period of HYK. The α-melanocyte stimulating hormone (α-MSH) is produced by pro-opiomelanocortin (POMC) neurons in the arcuate nucleus (ARC) of the hypothalamus. The α-MSH is an agonist of melanocortin 4 receptors (MC4Rs) and the melanocortin signaling mediates food intake, body weight and energy metabolism (Schwartz et al., 2000). The effect of *HTR2C* might be crucial for POMC neuronal activation (Xu et al., 2008). In addition, the activation of the central serotonin system has been linked with depressed appetite in almost all mammals (Tecott, 2007). Rodent models with a deletion of *HTR2C* showed hyperphagia and obesity (Xu et al., 2008). In humans, manipulation of 5-*HTR2C* receptors in the hypothalamus has been used to effectively induce weight loss using drugs such as d-fenfluramine and phentermine via blocking the reuptake of serotine and prompt its release (Zhou et al., 2005, Pissios and Maratos-Flier, 2007, Berglund et al., 2013). Moreover, HTR2C effect on POMC might be influenced by circulating energy metabolites such as glucose, fatty acids, leptin and insulin (Pissios and Maratos-Flier, 2007, Yadav et al., 2009).

ATP-binding cassette transporter ABCA1* and *ABCA2. ABCA1 is located on chromosome 8 from 96,274,035 - 96,390,357bp (RefSeq: NM_001024693.1). ***ABCA2*** (RefSeq: AC_000168.1), annotated in the human, mouse, and rat genomes, shows homology to this same region as well. In 5 out of the 8 performed GWAS, ***ABCA1*** and

ABCA2 appeared as candidate genes: Hyperketonemia (dichotomous), BHB-AUC (continuous), group 1 vs group 3 (dichotomous), group 1 vs group 4 (dichotomous), and group 2 vs group 4 (dichotomous). The adenosine triphosphate (ATP)-binding cassette (ABC) membrane transporter gene superfamily binds and hydrolyzes ATP to move different nutrients (amino acids, lipids, lipopolysaccharides, etc.) across the extracellular and intracellular membranes such as the endoplasmic reticulum (ER) (Dean et al., 2001; Attie, 2007), peroxisome and mitochondria (Kim et al., 2008, Szakács et al., 2008, Frikke-Schmidt, 2010). Mutations within the *ABCA1* gene in humans causes total or partial reduction of normal high-density lipoprotein (**HDL**) cholesterol due to the role of ABCA1 in HDL formation and in reverse cholesterol transport (Frikke-Schmidt et al., 2005, Kim et al., 2008). Subjects with this genetic variation accumulate cholesterol droplets in their liver, spleen, lymph nodes, intestine and nervous system (Frikke-Schmidt et al., 2004, Attie, 2007). In the same way, ABCA2 over expression has been correlated with decreased efflux and diminished esterification of lipoproteins (Davis, 2014). Functional annotation and the association of *ABCA1* and *ABCA2* to health disorders suggests that these genes may play a role in the movement of nutrients needed for energy metabolism and the possible build-up of lipids in the liver related to hyperketonemia.

Hepatic Lipase (LIPC). This gene is located on chromosome 10 from 51758867bp to 51921040bp (RefSeq: NM_001035410.1). In 4 out of the 8 performed GWAS, ***LIPC*** as a candidate gene: HYK (dichotomous), BHB-AUC (continuous), group 2 vs group 3 (dichotomous), and group 2 vs group 4 (dichotomous). The *LIPC* gene encodes for a lipolytic enzyme synthesized in the liver that plays a pivotal role in

several steps of lipoprotein metabolism (Perret et al., 2002, Xu et al., 2015). Up- and down-regulation have been associated with dyslipidemia; however, the pathways are not well understood (Rufibach et al., 2006). Cohen et al. (1994) estimated that the genetic variations at the *LIPC* gene might explain up to 25% of the total variation of HDL plasma concentrations in human monozygotic twins. Moreover, *LIPC* variants might have a pleiotropic effect on one more of the abnormalities associated with metabolic syndrome in humans such as insulin resistance (Kraja et al., 2011). *LIPC's* role in lipoprotein metabolism in the liver suggests it may be particularly relevant in the re-esterification of NEFA in the liver.

Heritability

The pseudo-heritability of HYK was 0.16±0.56 in our study and is congruent with a previous report of 0.17 (van der Drift et al., 2012), and greater than the heritability reported by others at 0.02 (Gröhn et al., 1984), and 0.06 (Uribe et al., 1995). Pseudo-heritability is defined as the fraction of phenotypic variance explained by the relationship matrix IBD. However, pseudo-heritability for some traits may over- or under-estimate heritability due to missing heritability, the proportion of genetic variance that cannot be explained by all significant SNPs (Zaitlen and Kraft, 2012, Korte and Farlow, 2013). The difference among these values could be attributed to the higher accuracy of disease characterization and its definition in the study of van der Drift et al. (2012) and our own. For our study, the pseudo-heritability of BHB-AUC (0.82±0.44) is a more rigorous estimate than the pseudo-heritability of HYK. The calculation used for pseudo-heritability of BHB-AUC included all serial measurements therefore the magnitude of standard error is smaller than that of HYK at one time point. While the

standard error and standard deviation of pseudo-heritability are high given our small sample size, the pseudo-heritability estimates provide a baseline to compare characteristics within the study (Zaitlen and Kraft, 2012).

A multitude of GWA studies have focused on production, phenotype, and health traits but only a small portion of these specifically investigate transition cows or tackle complex metabolic disorders. While our study had a relatively small sample size, reducing the power to find all true genomic associations, a similarly powered study of 73 individuals identified a QTL for Holstein cholesterol deficiency (Saleem et al., 2016) and an in-depth report on small sample size GWAS in dogs showed the effectiveness of just 20 dogs for mapping traits within breed (Karlsson et al., 2007). More importantly, the improved phenotypic characterization through serial measurements of NEFA and BHB concentration in blood during the first 16 DIM provides an advantage by allowing for a higher phenotypic reliability (Weigel et al., 2017). Indeed, simulation studies have found that a mere 10% misclassification of phenotype reduces the reliability of correctly identifying predictive SNPs in a GWAS to 54% (Smith et al., 2013, Rekaya et al., 2016). In addition, the use of a high-density SNP panel having markers spanning the genome is more desirable for identifying novel genomic regions associated with complex traits or diseases.

Frequently, there are multiple SNPs associated with complex diseases such as HYK and each one can increase the risk of developing the diseases in small increments. As understanding of HYK, energy metabolism, and the transition cow period is improved, we are afforded the opportunity to advance our knowledge of their genetic regulation. To date, one gene-based study combined with pathway analysis identified

various biological pathways associated with NEFA, BHBA and glucose changes in cows sampled 3 weeks before expected calving; 4 weeks postpartum, and 13 weeks after parturition (Ha et al., 2015). Preliminary data by Kroezen et al. (2016) identified a panel of 1,081 SNPs associated with HYK based on producer-recorded cases of clinical HYK to be tested in a larger cohort of Canadian cattle (Kroezen et al., 2016). Zoetis, a private company offering genotyping and genomic prediction services for dairy cattle, released their Wellness Traits, including ketosis predictions, in 2016 (McNeel et al., 2017). The Council on Dairy Cattle Breeding are now offering a similar genomic prediction for breeding merit of ketosis susceptibility as part of the U.S. National Dairy Cattle Genomic Evaluations as of April 2018 (Council on Dairy Cattle Breeding, 2017).

Despite these studies and efforts, the identification of QTL or genes influencing susceptibility to ketosis in dairy cattle have yet to be published. Our study differed from previous attempts to identify genomic regions associated with the development of HYK by using serial measurement data from dairy cows during the high-risk period of HYK, thus reducing the risk of phenotypic misclassification. Our positive results, despite a small cow cohort, demonstrates the informativeness of serial measures of BHB for the genomic analysis of hyperketonemia as supported by another recent study showing increased genomic prediction accuracy of HYK when using serial measures (Weigel et al., 2017). In all, the 5 novel candidate genes of *HSD17B10, HTR2C, ABCA1, ABCA2,* and *LIPC* were identified based on genome-wide association to either HYK status or serial blood concentrations of BHB and NEFA. Further confirmation of these regions and candidate genes using an unrelated population and/or expression studies is needed to establish the complete effect of them on HYK in early postpartum Holstein dairy

cows. The suggestive results for NEFA concentrations also warrant additional studies in a larger cohort of animals which may provide insight towards the genetic regulation of fat mobilization for energy metabolism. In all, this study provides a foundation to explore the genetic regulation of HYK and proposes markers for consideration in genomic selection schemes.

Conclusion

To conclude**,** HYK is one of the most important postpartum metabolic diseases in dairy cattle because of the negative association with reproduction, milk production, metabolic and infectious diseases and thus profitability and health of dairy cows. Our ability to identify the most susceptible animals has been constrained by the ability to measure metabolites such as BHB and NEFA during the high-risk period early postpartum when HYK is detected. Genetic studies have been similarly limited by the complexity of the phenotypic characterization of HYK. With these results and future validation in a larger population, the early identification of animals most susceptible to developing HYK using genomic information will provide producers with the ability to selectively breed for healthier animals and intensify the prophylactic measures for those deemed at risk.

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CHAPTER 3

EVALUATION OF THE DIAGNOSTIC ACCURACY OF TWO POINT-OF-CARE β-HYDROXYBUTYRATE DEVICES

Abstract

The use of point-of-care (POC) devices to measure blood metabolites, such as ß-hydroxybutyrate (BHB), on farm have become an important diagnostic and screening tool (Ospina et al., 2013) in the modern dairy industry. The POC devices allow for immediate decision-making and are often more economical than the use of laboratory-based methods but precision and accuracy may be lower when measurements are performed in an uncontrolled environment. Ideally, the advantages of the POC devices and the standardized laboratory environment could be combined when measuring samples that do not require an immediate result, for example in research applications or when immediate results are not necessary. The objective of this study was to compare the capability of two POC devices (TaiDoc, Pharmadoc, Lübeck, Germany; Precision Xtra, Abbott Diabetes Care Abingdon, UK) to measure BHB concentrations either at room temperature (RT; 20-22°C) or at 37°C compared with the gold standard test in stored plasma samples. Whole blood from multiparous Holstein dairy cows (n = 113) was sampled from the coccygeal vessels between 28 d before expected calving until 42 DIM. Whole blood BHB concentrations were determined cow-side using the TaiDoc POC device. Plasma was separated within 1h of collection and stored until analysis. A subset of stored plasma samples (n = 100) consisting of one sample per animal was chosen retrospectively based on the BHB concentrations in whole blood within the

range of 0.2 mmol/L to 4.0 mmol/L. The samples were analyzed for BHB plasma concentration using an automated chemistry analyzer (Hitachi 917, Hitachi, Tokyo, Japan) which was considered the gold standard. On the same day, the samples were also measured with the two POC devices with samples either at RT or heated up to 37°C. Our study showed high Spearman correlation coefficients (> 0.99) using either device and with samples at both temperatures compared with the gold standard. Passing and Bablok regression revealed a very strong correlation (> 0.99) indicating good agreement between both POC devices and the gold standard method. For hyperketonemia detection, defined as BHB concentration ≥1.2 mmol/L, the sensitivity for both POC devices at RT and 37°C was equally high at 100%. Specificity was lowest (67.4%) for the TaiDoc used with plasma at RT and was highest (86.5%) when plasma was measured at 37°C with the Precision Xtra meter. Bland-Altman plots revealed a mean bias of 0.25 mmol/L and 0.4 mmol/L for the Precision Xtra meter and TaiDoc, when tested on plasma at 37°C. Our data showed that both POC devices are suitable devices to measure BHB concentration in stored bovine plasma, and accuracy was highest when samples were heated to 37°C compared with RT.

Introduction

Subclinical hyperketonemia (**HYK**) in dairy cows has been defined as ß-hydroxybutyrate (BHB) concentration in blood of ≥ 1.2 mmol/L (Oetzel, 2004; McArt et al., 2011). The gold standard spectrophotometric test for the determination of BHB concentrations in either plasma or serum is performed under controlled laboratory and environmental conditions. Each test costs $13 USD (December 2017) at the New York Animal Health Diagnostic Center (**AHDC**, Ithaca NY). Consequently, a lower-cost method with comparable accuracy and precision to the gold standard is preferable for routine measurement of BHB plasma concentration in the dairy industry. Several point-of-care (**POC**) devices were previously validated as cow side methods to measure BHB concentration in bovine whole blood (Iwersen et al., 2009; Bach et al., 2016). Because of their user-friendliness, low maintenance and calibration requirements, POC devices represent valuable tools in the field. Moreover, such a method may allow retrospective sample analysis under controlled environmental conditions, for example in research applications (Pineda and Cardoso, 2015) or when immediate results are not needed. Often, plasma is the sample of choice for storage, but available POC devices are calibrated for use on whole blood, requiring validation of the use of this sample type in such applications. The Precision Xtra (Abbott Diabetes Care, Abingdon, UK) has been widely used as a diagnostic tool for HYK in the field (McArt et al., 2011; Mann et al., 2015) and was recently validated as a feasible method to estimate BHB concentration in bovine plasma and serum (Pineda and Cardoso, 2015). The approximate cost for each BHB test using the Precision Xtra was $1.70 to $4.00 USD (Leblanc, 2010), plus the cost of the meter. Currently, the Precision Xtra test strips are only available and sold for

human use in the US, increasing the cost and reducing access for veterinary applications.

Recently, the TaiDoc POC device (Pharmadoc, Lübeck, Germany) was shown to measure BHB concentration in bovine whole blood with good accuracy and precision when testing was performed at room temperature (Bach et al., 2016). The current cost for each TaiDoc strip is approximately $1.10 USD and makes this a convenient and cost-effective method for BHB testing. However, the temperature range for the TaiDoc meter is 5 to 40°C and is similar to the Precision Xtra (10 to 50°C). Using POC devices on-farm under conditions that differ from manufacturer specifications may often lead to unknown variation in accuracy and precision due to limitations in temperature, humidity and sample quality. A possible solution is to combine the ease and cost-effectiveness of POC devices with analysis in a controlled environment after sample collection. Plasma samples are easily obtained and BHB concentrations are stable in this sample type (Stokol and Nydam, 2005).

The objective of this study was to compare the results of the TaiDoc POC device for BHB concentrations in plasma either at room temperature (RT) or at 37°C compared with the results obtained with the gold standard method and a second POC device previously described for use in plasma samples (Precision Xtra; Pineda and Cardoso, 2015). Room temperature was tested in addition to 37°C as laboratory tests are often carried out at either of the two temperatures. The 37°C temperature was selected to reproduce closely the conditions when using fresh blood samples on POC devices, as well as to approximate the gold standard methodology.

Materials and methods

All procedures for this experiment were approved by the Cornell University Institutional Animal Care and Use Committee (Protocols number 2014-0118 and 2015-0097). Holstein dairy cows (n = 113) from the Cornell University Ruminant Center located in Harford, NY were enrolled between January 2016 and July 2016. All cows were entering second or greater lactation and were sampled from 28 d before expected calving date until 42 DIM. Whole blood was collected three times per week from the coccygeal vessels using 10 ml blood collection tubes (Becton, Dickinson and Company Becton Drive Franklin Lakes, New Jersey) containing 158 USP of sodium heparin and 20-gauge x 2.54 cm blood collection needles. Concentration of BHB was measured immediately after sample collection in whole blood using the TaiDoc POC device (Bach et al., 2016). After measurements were completed, blood samples were placed on ice and plasma was separated within 1 h of sample collection at 3,000 x *g* for 20 min at 4°C and stored in aliquots at -20°C until analysis.

A subset of plasma samples (n = 100) was chosen retrospectively based on the BHB concentrations in whole blood obtained with the TaiDoc POC device. Samples were included in this study based on whole blood BHB concentrations in 0.1 mmol/L increments ranging from 0.2 mmol/L to 4.0 mmol/L with no more than 4 samples per increment. The plasma sample subset did not include more than one sample per animal to assure independence of observations for statistical analysis. Samples were allowed to thaw on ice, and submitted to the AHDC for measurement of BHB concentration using a commercially available test kit (D-3 Hydroxybutyrate Ranbut, Randox Laboratories, Antrim, UK) on an automated chemistry analyzer at 37 ± 0.2 °C (Hitachi 917, Hitachi,

Tokyo, Japan) as the gold standard. The BHB plasma concentrations were measured on the same day as the gold standard and on the same aliquot with both POC devices as submitted to the AHDC. Meters were used at all times at room temperature (RT; 20-22°C). Plasma samples were measured first at RT with both devices and then at 37°C after heating plasma samples for at least 5 minutes in a water bath.

Statistical analysis

Data analysis was performed in SAS (SAS 9.4, SAS Institute Inc., Cary, NC). Correlation coefficients (Spearman) between BHB gold standard and BHB concentrations obtained with both POC devices, and samples either at RT or 37°C, were computed using Proc Corr (SAS 9.4). Coefficient of variation (CV, %) was determined for 1 sample each in the low (0.8 mmol/L) and high (3.9 mmol/L) range of our data based on the gold standard method. For each sample 12 measurements were performed with each POC device, and with the sample either at RT or 37°C. Sensitivity of each method was calculated as the proportion of animals properly diagnosed as positive for HKY (BHB plasma concentration ≥ 1.2 mmol/L) among all animals identified as positive by each POC devices compared with those classified as positive by the gold standard. Specificity was calculated as the proportion of animals properly diagnosed as negative for HKY among all animals identified as negative by each POC device compared with those classified as negative by the gold standard. The 95% CI were calculated for sensitivity and specificity values using Proc FREQ in SAS. Regression analysis between both methods with samples at RT and 37°C and the gold standard were performed using Proc REG in SAS. Linearity between methods were graphically assessed using the plot of residuals. Passing and Bablok regression coefficients, slopes,

and intercepts were obtained using MedCalc Statistical Software version 17.8.6 (MedCalc Software bvba, Ostend, Belgium; 2017). This regression fits a straight line to two variables and it is less sensitive to outliers because it assumes measurement errors in both methods. Perfect agreement yields an intercept of 0 and a slope of 1. Bland-Altman plots were generated with GraphPad Prism (v. 7.02, La Jolla, CA) to graphically demonstrate the level of agreement between two tests which cannot be captured by the correlation estimate alone (Bland and Altman, 1986). The Bland-Altman plot includes a solid horizontal line showing the mean bias between the two methods, as well as the 95% confidence interval of agreement.

Results and discussion

Out of the 100 plasma samples used in this experiment, 57 had a BHB plasma concentration ≥ 1.2 mmol/L as assessed by the gold standard method. Data of BHB plasma concentrations from the gold standard test showed a non-parametric distribution. The median was 1.3 mmol/L, ranging from 0.1 mmol/L to 5.5 mmol/L. Spearman correlation coefficients of the measured BHB plasma concentrations between the gold standard and both POC devices at both plasma temperatures were > 0.99. Similar coefficients of correlation were reported for the Precision Xtra (Pearson r = 0.99) on bovine plasma (Pineda and Cardoso, 2015) and for the Optium Xceed POC meter (Abbott Diabetes Care, Abingdon, UK) in K_3-EDTA anticoagulated bovine whole blood compared with analysis of plasma using the laboratory gold standard method (Pearson r = 0.97) (Voyvoda and Erdogan, 2010). Additionally, the correlation coefficients in our study for both POC devices in plasma either at RT or at 37°C were comparable to other studies using whole blood, for example 0.95 (Iwersen et al., 2009) and 0.97 (Bach et al.,

2016). Coefficients of variation were generally lower when samples were heated up to 37°C. The CV for the sample at 0.8 mmol/L was 11, 4, 12, and 9% for TaiDoc with plasma at RT, TaiDoc with plasma at 37°C, Precision Xtra with plasma at RT, and Precision Xtra with plasma at 37°C, respectively. The CV for the sample at 3.9 mmol/L was 2, 2, 7, and 5 % for the two devices and temperatures, respectively.

Sensitivity and specificity of both POC devices for the classification of HYK are summarized in Table 3.1. The TaiDoc and Precision Xtra measurements of plasma at both RT and 37°C had perfect sensitivity for HYK. The greatest specificity for HYK was shown for Precision Xtra and TaiDoc at 37°C sample temperature. A higher sensitivity for Precision Xtra and TaiDoc meters was demonstrated in this study compared with a sensitivity of 0.85 described by (Voyvoda and Erdogan, 2010), but similar to other studies using bovine whole blood, plasma, and serum (Iwersen et al., 2009; Pineda and Cardoso, 2015; Bach et al., 2016). Moreover, specificity values presented here were higher than the specificity of 0.51 previously reported by (Pineda and Cardoso, 2015), but were lower than the values found in several other studies (Iwersen et al., 2009; Voyvoda and Erdogan, 2010). The temperature of whole blood, serum and plasma can affect the results for the concentration of metabolites such as nonesterified fatty acid (Stokol and Nydam, 2005), glucose (Megahed et al., 2015) and BHB (Iwersen et al., 2013; Megahed et al., 2015). This may also be the reason for the superior CV results for samples heated up to 37°C in this study. Differences in sensitivity and specificity may be attributed to different temperatures in our and other studies. It has been shown that when POC devices and strips are exposed to temperatures out of the range specified by the manufacturer erroneous measurements might result

(Nerhus et al., 2011; Deakin et al., 2015).

Table 3.1. Performance of two point-of-care-test devices for the classification of hyperketonemia, defined as ß-hydroxybutyrate plasma concentration ≥ 1.2 mmol/L at room temperature or 37°C (n=100).

Test[1]	Plasma Temperature[2]	Sensitivity[3] (%) (95% CI)	Specificity[4] (%) (95% CI)
TaiDoc	RT	100.0 (93.7, 100.0)	67.4 (51.4, 80.9)
TaiDoc	37ºC	100.0 (93.7, 100.0)	76.4 (61.3, 88.2)
Precision Xtra	RT	100.0 (93.7, 100.0)	74.4 (58.8, 86.4)
Precision Xtra	37ºC	100.0 (93.7, 100.0)	86.5 (72.0, 94.7)

[1] TaiDoc (Pharmadoc, Lübeck, Germany) and Precision Xtra (Abbott Diabetes Care Abingdon, UK) devices

[2] BHB concentration was measured in the same aliquot of plasma at room temperature (RT; 20-22°C) and at 37°C;

[3]Sensitivity was calculated as the proportion of animals that were classified as positive for hyperketonemia by the meter compared with the animals classified as positive with the gold standard method (BHB plasma concentration ≥1.2 mmol/L) performed at the New York Animal Health Diagnostic Center (**AHDC**, Ithaca NY)

[4]Specificity was calculated as the proportion of animals that were classified as negative for hyperketonemia by the meter as compared with the number of animals classified as negative by the gold standard method (BHB plasma concentration <1.2 mmol/L) performed at the New York Animal Health Diagnostic Center (**AHDC**, Ithaca NY)

Linear regression is traditionally performed when comparing two methods with continuous outcomes, but this technique assumes normality of data distribution, errors, as well as absence of error in the gold standard. Linear regression analysis revealed a linear relationship between both methods with plasma at RT and 37°C, and the gold standard (Figure 3.1). The TaiDoc meter used with plasma at 37°C had the greatest linear relationship with the gold standard test ($R^2 = 0.97$). However, when linearity and normality were graphically assessed using the plot of residuals, a deviation from both linearity and normality were observed suggesting lack of fit for all linear regression models, making this a less desirable technique for method comparison in this case. This can be caused by random error within both the gold standard method and POC devices or intermittent imprecision from the POC devices (Stöckl et al., 1998). Therefore, we explored Passing and Bablok regression because this allows us to compare methods with non-parametric distributed data (Table 3.2); however, this method also assumes linearity between the two variables. The intercept is interpreted as the systematic mean bias between the two methods, whereas the slope indicates proportional bias between two methods. The Spearman's correlation coefficients obtained from the Passing and Bablok regressions showed a strong correlation (> 0.99) between the gold standard and the two POC devices. According to (Passing and Bablok, 1983), there is no constant difference between the two methods if the 95% confidence interval of the intercept includes zero. Therefore, among all comparisons the TaiDoc in plasma at 37°C (intercept 0.05; 95% CI: -0.01, 0.11) was the only POC device without a constant difference compared with the gold standard.

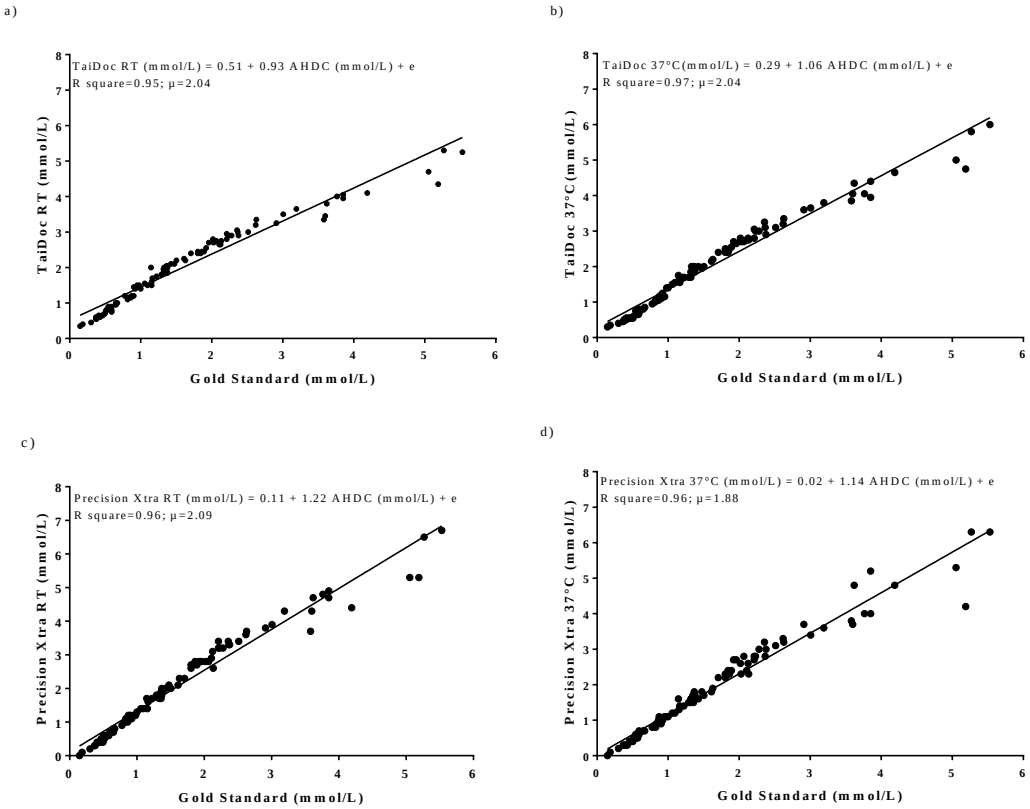

Figure 3.1. Linear regression analysis between BHB concentrations measured in plasma with the gold standard method (Randox Laboratories, Antrim, UK) and two point-of-care devices at different temperatures. a) TaiDoc (Pharmadoc, Lübeck, Germany) in plasma at room temperature (**RT**; 20-22°C); b) TaiDoc measured in plasma at 37°C; c) Precision Xtra (Abbott Diabetes Care Abingdon, UK) in plasma at RT; d) Precision Xtra in plasma at 37°C. The values obtained by New York Animal Health Diagnostic Center (AHDC) were used as the gold standard test. μ is the mean of all BHB concentrations measured in plasma with each device.

Table 3.2. Passing and Bablok regression coefficients for ß-hydroxybutyrate concentrations in 100 plasma samples at room temperature or 37°C measured with two different POC devices compared with the gold standard test performed at the New York Animal Health Diagnostic Center (AHDC, Ithaca NY)

Test[1]	Plasma Temperature[2]	Intercept (95% CI)	Slope (95% CI)
TaiDoc	RT	0.22 (0.16, 0.30)	1.17 (1.10, 1.20)
TaiDoc	37ºC	0.05 (-0.01, 0.11)	1.26 (1.20, 1.32)
Precision Xtra	RT	-0.19 (-0.23, -0.14)	1.46 (1.41, 1.50)
Precision Xtra	37ºC	-0.18 (-0.21, -0.14)	1.31 (1.26, 1.34)

[1] TaiDoc (Pharmadoc, Lübeck, Germany) and Precision Xtra (Abbott Diabetes Care Abingdon, UK) devices

[2] BHB concentration was measured in the same aliquot of plasma at room temperature (**RT**; 20-22°C) and at 37°C.

The test agreement between BHB plasma concentrations measured by the gold standard and both POC devices is illustrated in Figure 3.2. The Precision Xtra and the TaiDoc devices with samples at 37°C had a mean bias (i.e. the mean difference in accuracy of the tested methods compared with the gold standard) of 0.25 mmol/L (95% CI: -0.38, 0.89,) and 0.40 mmol/L (95% CI: -0.99, 0.99), respectively when compared with the gold standard. Although the TaiDoc had a larger bias, the 95% confidence limits of agreement for TaiDoc on plasma at 37°C were smaller than those of the Precision Xtra. The mean bias, that we determined for both POC devices in this study was in agreement with prior reports using Precision Xtra in bovine plasma (Voyvoda and Erdogan, 2010; Pineda and Cardoso, 2015; Bach et al., 2016). Users of POC devices should be aware of the bias in BHB concentrations compared with the gold standard, particularly when BHB concentrations increase. For both meters and either sample temperature, the bias was positive, meaning that BHB concentrations are

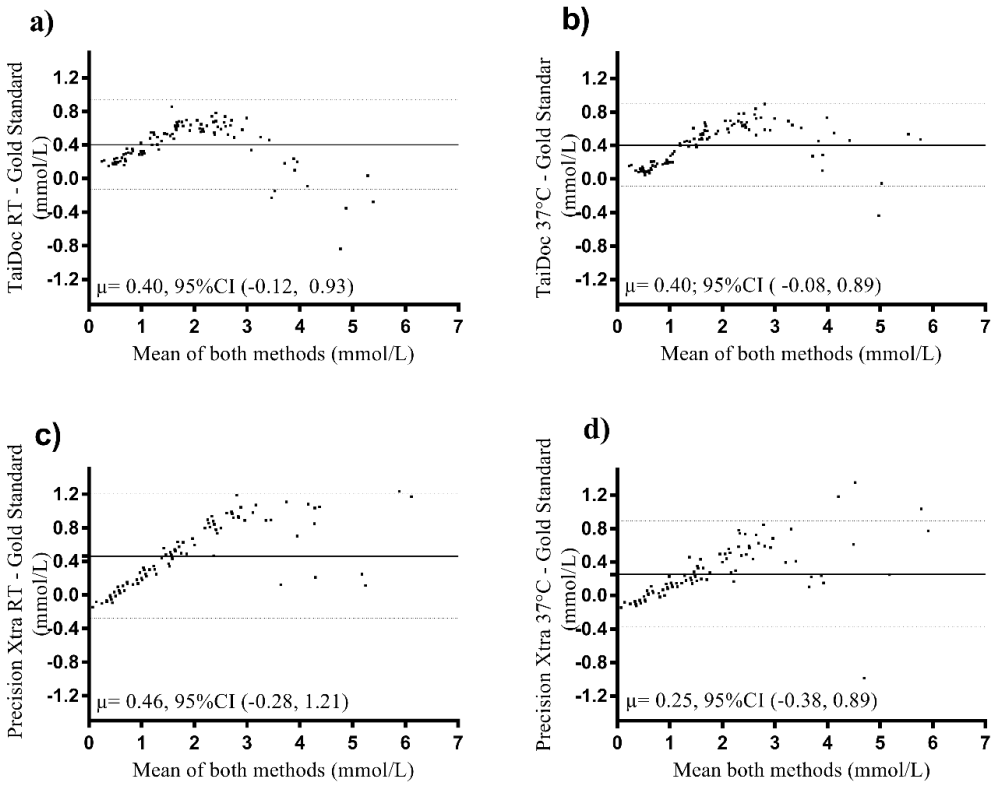

Figure 3.2. **Bland-Altman plots of the difference in BHB concentrations in plasma between two tests against their mean.** a) TaiDoc (Pharmadoc, Lübeck, Germany) at room temperature (RT; 20-22°C) vs. gold standard; b) TaiDoc at 37°C vs. gold standard; c) Precision Xtra (Abbott Diabetes Care Abingdon, UK) at room temperature (RT) vs. gold standard; and d) Precision Xtra at 37°C vs. gold standard. The solid horizontal line represents the mean bias; horizontal dashed lines represent the 95% confidence interval of agreement. μ is the overall mean bias (mmol/L) calculated as POC device (either TaiDoc or PrecisionXtra) – gold standard. A positive mean bias shows an overestimation of the BHB concentration by the POC device compared with the gold standard.

generally overestimated by these methods compared with the gold standard. The mean bias for the TaiDoc and Precision Xtra devices was similar to the values reported recently for whole blood samples (0.34 and 0.21 mmol/L, respectively). However, given that the method comparisons showed deviation from linearity, samples in the low- to midrange of BHB concentration were overestimated for the TaiDoc, whereas samples

with the highest BHB concentrations are underestimated by both POC devices. In contrast, measurements of BHB in plasma with the Precision Xtra seemed to consistently overestimate concentrations, with the bias increasing as concentrations measured with the gold standard increased.

Based on the sensitivity and specificity, the tested POC devices can be used with satisfactory accuracy to classify samples in hyperketonemic or non-hyperketonemic in bovine plasma when measured at 37°C. The precision of both devices was higher with samples at 37°C based compared with RT based on the CV. Mean bias was comparable to results previously reported in whole blood for both meters at both temperatures. When POC devices are used, sample temperature plays an important role. In conclusion, this work provides evidence that POC devices can be used with stored plasma samples. This enables the use of POC devices in research applications when such samples are available for BHB measurements instead of fresh whole blood.

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CHAPTER 4

RUMEN-PROTECTED BRANCED-CHAIN AMINO ACID SUPPLEMENTATION DURING EARLY LACTATION

Abstract

Essential AA (**EAA**) are critical for multiple physiological processes. Branched-chain amino acid (**BCAA**) supplementation has beneficial effects on body weight, fat tissue and insulin resistance in several species. The BCAA are used for milk and body protein synthesis as well as oxidized by the tricarboxylic acid cycle to produce ATP during catabolic states. The objective was to evaluate the effect of rumen protected branched Fchain AA (**BCAA;** 375 g of 27% L-Leu, 85 g of 48 % L-Ile and 91 g of 67% L-Val) with or without propylene glycol (**PG**) oral administration on milk production, dry matter intake, free fatty acids (**FFA**), β-hydroxybutyrate (**BHB**), and plasma urea N (**PUN)** during the early postpartum period in dairy cattle. Multiparous Holstein cows were enrolled in blocks of three and assigned randomly to either the control group or one of two treatments from calving until 35 DIM. The control group (n=26) received 200 g/d of dry molasses; the BCAA group (n=23) received BCAA mixed with 200 g/d of dry molasses; the BCAA plus PG (BCAAPG) group (n=25) received BCAA mixed with 200 g/d of dry molasses plus 300 ml of PG once daily from calving until 7 DIM. Postpartum, dry matter intake (DMI) average (95% CI) was 20.7 (19.9, 21.7), 21.3 (20.4, 22.3) and 21.9 (20.9, 22.8) kg for control, BCAA and BCAAPG, correspondingly. Milk yield (95%CI) was 41.7 (39.4, 44.0), 42.7 (40.3, 45.0) and 43.7 (41.4, 46.0) kg for control, BCAA and BCAAPG, respectively. ECM (95% CI) was 50.3

(46.8, 53.7), 52.4 (48.9, 55.8) and 52.9 (49.5, 56.4) kg for control, BCAA and BCAAPG, respectively. Milk urea nitrogen (% DM) in milk for control, BCAA and BCAAPG were 8.60 (8.02, 9.22) %, 9.70 (9.01, 10.45) % and 9.75 (9.08, 10.47) %. Plasma urea nitrogen concentrations (95% CI) for control, BCAA and BCAAPG were 8.3 (7.7, 8.9), 10.1 (9.4, 10.9), and 9.6 (9.4, 10.3) mg/dL, respectively. The number of plasma samples classified as HYK was 77, 44 and 57 in group control, BCAA and BCAAPG, respectively The BCAA supplementation increased PUN and MUN, free valine concentration in plasma and decreased hyperketonemia events during the postpartum period.

Introduction

There are hundreds of amino acids (AA) in nature but only the few known as essential (EAA) cannot be synthesized by the mammalian tissues and are basic building blocks for the synthesis of proteins. For this reason, these EAA must be provided in the diet to meet AA daily requirements. Deficiencies in EAA metabolism may disrupt homeostasis in the dairy cow and affect all physiological functions such as maintenance, growth, reproduction and lactation (Bauman and Currie, 1980, Orlando et al., 2008). During late gestation and early lactation, the dairy cow experiences an increased demand for protein to support fetal development, protein synthesis in the mammary gland and in other body tissues (Drackley, 1999, Ji and Dann, 2013, Van Saun and Sniffen, 2014). The deficit in protein intake is counterbalanced by increasing protein mobilization from body reserves such as skeletal muscle (Bell et al., 2000, Kuhla et al., 2011, Mann et al., 2016). The efficiency of energy use from oxidation of EAA to ATP ranges from 29% for Met to 59% for Ile (Wu, 2009). Leucine, isoleucine and valine, also known as branched-chain AA (BCAA), are three of the ten known EAA. The BCAA are used for cellular and milk protein synthesis and represent a large percentage of all EAA in milk and muscle protein (Harper et al., 1984, Mackle et al., 1999a, Appuhamy et al., 2011). Larsen et al. (2014) reported an increased milk yield 7.2 kg/d and lactose yield of 436 g/d during early lactation in dairy cows by infusing casein into the abomasum from calving until 20 DIM. This author also proposed that Leu would be one of the two most limiting EAA during early postpartum based on the concentration differences of AA between mammary arterial and venous blood. The effects of BCAA supplementation on glucose homeostasis and positive regulation of AA have been

reported in several species (Shimomura et al., 2006, Torres-Leal et al., 2011, Lynch and Adams, 2014). Moreover, increased levels of circulating BCAA are potent nutrient signals that stimulate the mammalian target of rapamycin (mTORC) pathway inducing increased protein synthesis and influence the hormonal status during catabolic stages such as in early lactation dairy cows (Lynch and Adams, 2014, Yoon, 2016).

The use of propylene glycol (PG) as a treatment for hyperketonemia (HYK) has been described previously (Hoedemaker et al., 2004, McArt et al., 2011, Mann et al., 2017). Hyperketonemia is one of the most common metabolic disorders during early lactation in dairy cows due to the reduced dry matter intake and increased demand of nutrients (Duffield et al., 2009, Ospina et al., 2010). Propylene glycol is fermented to propionate or lactic acid, essential substrates for gluconeogenesis, and then used for synthesis of glucose by the liver. The use of PG might help to overcome energy deficit and diminish use of body reserves for gluconeogenesis during early lactation (Studer et al., 1993, Drackley et al., 2001, Nielsen and Ingvartsen, 2004). PG as an oral drench has been shown to increase insulin and glucose concentrations in plasma (Studer et al., 1993, Miyoshi et al., 2001, Piantoni and Allen, 2015), as well as increase milk production, and decrease clinical diseases, and culling in HYK cows (McArt et al. 2012). Increased concentration of insulin stimulates AA disposal into body tissues (Davis et al., 2002) and drastically reduces the concentration of free BCAA in blood (Mackle et al., 1999b), suggesting an increased use by peripheral tissues. Thus, synthesis and degradation of AA could be altered after a bolus of PG and its subsequent effects on insulin and glucose metabolism during catabolic stages (Eriksson and Björkman, 1993).

The positive effects of BCAA supplementation on milk protein yield in dairy

cows have been reported before by several authors (Appuhamy et al., 2011, Doelman et al., 2015). These studies used either post-ruminal or intravenous infusion of BCAA, predominantly in mid to late lactation animals (Mackle et al., 1999a, Korhonen et al., 2002, Nichols et al., 2016). However, infusion and manipulation of dietary BCAA produced ambiguous results in milk yield and other milk components such as milk fat concentration and lactose in dairy cows (Hopkins et al., 1994, Arriola Apelo et al., 2014, Curtis et al., 2018).

We hypothesize that BCAA supplementation alone or in combination with PG orally during early lactation in dairy cows has a beneficial role in milk yield and protein synthesis in the mammary gland as well as altering energy and protein blood metabolites associated with catabolic processes. To the best of our knowledge this is the first study using rumen protected branched chain AA (**BCAA**) as a practical strategy to test the potential benefits of BCAA supplementation during early lactation in Holstein dairy cows. The objective of our study was to test dietary supplementation of BCAA with or without PG oral administration in order to evaluate the effect on milk yield, ECM, milk composition, energy balance (**EB**), protein balance (**PB**), and energy related plasma metabolites during the early postpartum period in dairy cows.

Materials and methods

Study population, diets and treatments

All procedures were approved by the Cornell University Institutional Animal Care and Use Committee (protocol # 2011-0016). Holstein dairy cows (n= 81) from the Cornell University Ruminant Center (Harford, NY) were enrolled between January and

July 2016. Inclusion criteria for enrollment were cows entering second or greater lactation and having no history of chronic lameness or mastitis during the previous lactation. At enrollment, cows were blocked based upon their expected calving date then assigned randomly using a statistical software (PROC PLAN; SAS 9.4, SAS Institute Inc., Cary, NC) into one of the 3 treatment groups: control (n=27) received 200 g/d of dry molasses only from calving to 35 DIM; branched chain AA (**BCCA)** (n=27) received 550 g/d of BCAA with 200g of dry molasses from calving to 35 DIM; or branched chain AA plus propylene glycol (**BCAAPG**) (n=27) received 550 g/d of BCAA with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

The respective BCAA treatment or dry molasses was offered daily as a top-dress supplement immediately after the TMR was placed in the individual bins. The BCAA was composed of 375 g (27 % wt./wt.) rumen protected L-Leu, 85 g (48 % wt./wt.) rumen protected L-Ile and 91 g of (67 % wt./wt.) rumen protected L-Val. The chemical composition of the BCAA is presented in Table 4.1. The BCAA were protected to prevent ruminal degradation using a lipid coating layer with soy based hydrogenated vegetable oil, (Balchem Corporation, New Hampton, NY). The intended dose of BCAA in this study was calculated based on previous studies that effectively increased free BCAA in blood using post ruminal infusion of BCAA or casein (Mackle et al., 1999a, Larsen et al., 2014). Due to discrepancies in the rumen protected L-Leu concentration, cows in the first 10 enrollment blocks of the study received 187 g of L-Leu, whereas cows in blocks 11 to 27 received 375 g of this AA. To account for this difference and other changes associated with these two distinct time periods of the study (i.e. diet

composition and ambient temperature), a fixed effect of period was included in all models, and interactions with all other fixed effects were evaluated.

Table 4.1. Chemical composition of the rumen protected branched-chain amino acids (%DM).

	Rumen protected branched-chain amino acids[1]		
	L-leucine[2]	L-valine[3]	L-isoleucine[4]
Chemical composition			
DM %	99	99	100
CP % DM	27	67	48
Soluble protein	9.1	31.9	36.4
ADF % DM	12.3	1.9	6.7
aNDF % DM	15.3	3	14.6
Starch % DM	0.1	0.1	0.1
Ether extract % DM	58.1	23.9	37.6
Ash % DM	0.46	0.37	0.71
Ca % DM	0.01	0	0
P % DM	0	0	0
Mg % DM	0	0	0
K % DM	0.01	0.1	0.01
S % DM	0.020	0.1	0.01
Na % DM	0	0	0
Cl % DM	0	0	0

[1]Chemical composition was obteined with by Wet Chemestry (Cumberland Valley Analytical Services, Waynesboro, PA).
[2]L-leucine 27 % rumen protected Balchem™ Corporation, New Hampton, NY.
[3]L-valine 67 % rumen protected Balchem™ Corporation, New Hampton, NY.
[4]L-isoleucine 48 % rumen protected Balchem™ Corporation, New Hampton, NY.

Cows were fed for ad libitum intake to achieve a minimum of 5% refusals. All cows were kept in sawdust bedded tie stalls and fed the same close-up dry period and fresh period TMR. Prepartum and postpartum diets were formulated with the Cornell Net Carbohydrate and Protein System software (CNCPS; Cornell University, version 6.55), Table 4.2. Throughout the study, fresh samples of prepartum and postpartum TMR were collected weekly and submitted fresh for analysis by near infrared reflectance spectroscopy (NIR; Cumberland Valley Analytical Services, Waynesboro, PA). Weekly samples from all forages and concentrate grains were dried, ground, and a single composite of each ingredient was analyzed for DM (method 930.15; AOAC International, 2000), CP (method 990.03; AOAC International, 2000), soluble protein (Krishnamoorthy et al., 1982), ADF (method 973.18; AOAC International, 2000), NDF (Van Soest et al., 1991), starch (Hall, 2009), sugar (DuBois et al., 1956), ash (method 942.05; AOAC International, 2000), and minerals (method 985.01; AOAC International, 2000). The grain composition as formulated and an average of the weekly forage analysis were entered into the CNCPS v. 6.55 feed library to obtain predicted energy and protein content of all rations in the course of the study. Protein and energy balance as percentage were calculated with CNCPS v. 6.55 for each cow during the prepartum and postpartum periods using a weekly average of BW, DMI, and either days carried calf or milk yield and milk components. Final crude protein across all treatments was calculated with CNCPS v. 6.55 as percentage of the DM using an average of the weekly feed samples. Results were similar across groups; 15.6 %, 16.0 % and 15.8 % for control, BCAA and BCAAPG, respectively. The duodenal flow of EAA was calculated using the mean of the DMI for each treatment group on CNCPS v. 6.5.

Table 4.2. Ingredients of dry and fresh diet formulation (% of DM).

	Dry	Fresh
Ingredient		
Wheat straw	21.33	3.38
Corn silage	46.21	43.19
Haylage	-	6.57
Alfalfa hay	-	3.76
Amino Plus[1]	7.11	5.66
Canola meal, solvent extracted	3.56	6.95
Corn grain, ground fine	-	14.12
Citrus pulp	4.62	1.87
Soybean hulls	3.56	3.65
Corn gluten feed	1.78	-
Megalac R[2]	0.89	0.43
Urea		0.21
Salt	-	0.42
Bio-Chlor[3]	5.33	-
Sodium bicarbonate	-	1.33
Calcium carbonate	2.31	0.17
Calcium phosphate	-	0.17
Magnesium sulfate	0.36	-
Magnesium oxide	0.44	0.21
Alimet[4]	0.11	0.06
Megamine-L[5]	-	0.63
LysAAmet[6]	1.07	1.60
Organic TM[7]	0.44	0.21
Rumensin[8]	0.09	0.06
MIN-AD[9]	-	0.42
Dynamate[10]	-	0.44
Vitamin E	0.27	0.04
Organic selenium 0.06%	0.52	-

[1]Soybean product, Ag Processing Inc, Omaha, NE

[2]Comercial fat, Arm & Hammer Nutrition, Princeton, NJ

[3]Anionic feed supplement, Arm & Hammer Nutrition, Princeton, NJ

[4]2-hydroxy-4-methyl-thio-butanoic acid, Novus International, St. Charles, MO

[5]Rumen bypass lysine supplement, Arm & Hammer Nutrition, Princeton, NJ

Sample collection

Cows were enrolled 28 d before expected calving date, allowing them one week of adaptation before the sampling period started. Weekly, BW was determined after morning milking and BCS was assessed using a 1-5 scale (Edmonson et al., 1989). Changes in BW were calculated as the BW 1 wk postpartum minus the BW at 6 wk postpartum. Calving ease on a scale 1 to 5 (1 = normal or no assistance; 2 = moderate assistance, provided by farm staff; 3 = moderate assistance, but vet called as a precaution; 4 = difficult calving, with extraction done by skilled farm staff; 5 = very difficult calving, with maximum veterinary assistance) was recorded by farm personnel.

Postpartum, cows were milked three times per day and milk weights were recorded at each milking. Milk samples were collected from three consecutive milkings once per week, stored with bronopol at 4°C, then analyzed within 24 h for fat, true protein, lactose, total solids and MUN using infrared analysis on an automated Fossomatic FT+ (Foss, Eden Prairie, MN; method 972.160; AOAC International, 2012), and SCC using optical fluorescence on a Fossomatic FC (method 972.160; AOAC International, 2012; Dairy One Cooperative Inc., Ithaca, NY). Linear scores (**LS**) were calculated as: LS = [ln (SCC/100)/ln(2)]+3 (Ali and Shook, 1980). Energy corrected milk yield was calculated for 3.5% fat and 3.0% protein as follows: ECM (kg) = {[(0.0929 × fat %) + (0.0563 × true protein %) + 0.192] × milk (kg) / 068605} (Mann et al., 2015).

Blood was sampled 3 times per week from 21 d before expected calving until 21 DIM from the coccygeal vessels using 20 x 2.54 cm needles and blood collection tubes (Becton, Dickinson and Company Becton Drive Franklin Lakes, New Jersey)

containing 158 USP of sodium heparin and without anticoagulant, for plasma and serum separation. All blood samples were immediately placed on ice and plasma and serum were separated within 1 h at 2,800 × *g* for 20 min at 4°C, then stored at -20° until analysis.

Free fatty acid (FFA) concentrations in plasma were estimated by colorimetric measurement of an enzymatic reaction (HR Series NEFA-HR (2); Wako Life Sciences, Mountain View, CA) with a microplate spectrophotometer (Epoch, Biotek, Winooski, VT) as previously described (Mann et al., 2015). Plasma urea nitrogen (**PUN**) concentrations were established using a manual urease/Berhelot determination (Sigma, urea nitrogen procedure no. 640, Sigma Diagnostics, St. Louis, MO) (Butler et al., 1996). For quality control, in-house pooled bovine quality control samples were included on each FFA and PUN plate.

The plasma BHB concentration was measured using the BHB-Check Ketone meter system (Pharma DOC, Lübeck, Germany) in samples thawed on ice and then warmed up briefly in a water bath at 37°C (Leal Yepes et al., 2018). HYK was defined as BHB ≥ 1.2 mmol/L (Ospina et al., 2013). Cows were not treated for HYK unless contemporaneous with anorexia, upon which they were removed from the trial and treated according to standard farm protocols, (BCAA n=1). Health events were recorded on a daily basis for all cows during the study period.

Plasma free AA concentrations were established using EZ:faast (Phenomenex, Torrance, USA) by the chromatography and diagnostic services laboratory of the Veterinary faculty, University of Montréal as previously described (Kassube et al., 2017). Briefly, solid phase extraction was followed by derivatization and a liquid/liquid

extraction. Derivatized samples were then analyzed by liquid chromatography-mass spectrometry using the Agilent 6100 Single Quadrupole LC/MS Systems for high-throughput qualitative analyses of small molecules. For a separate objective of this study that included liver, muscle, and adipose tissue biopsies, cows received flunixin meglumine (1.1 mg/kg, IV Prevail, VetOne) intravenously at d 9 ± 4 pre-partum and d 5 ± 1, and d 20 ±1 postpartum (data not presented).

Statistical analysis

Prior to any statistical analysis, seven cows were removed from the study for the following reasons: one animal (BCAA n=1) died as a consequence of an uterine prolapse; one animal (BCAA n=1) was removed from trial due to respiratory disease; one animal (Control n=1) was unable to adapt to the tie stall; one animal (BCAAPG n=1) had an incorrect breeding date; one animal (BCAA n =1) was anorexic for several days after calving and, two animals (BCAA n=1; BCAAPG n=1) were treated for suspected hepatic lipidosis following the farm's protocols, confounding the data. The number of cows remained for statistical analysis were control (n=26), BCAA (n=23) and, BCAAPG (n=25).

Weekly averages for DMI and milk yield were calculated first and used for analysis. Chi-squared tests were performed using PROC FREQ of SAS (SAS 9.4, SAS Institute Inc., Cary, NC) for differences in calving scores, parity, and episodes of HYK (BHB ≥ 1.2 mmol/L). One way ANOVA were calculated using PROC ANOVA in SAS 9.4 for differences in days dry, BCS and BW. Repeated measures ANOVA was performed for the outcomes: DMI, EB, PB, milk yield, ECM yield; percentage of fat, protein, lactose and total solids; MUN, LS, free AA, BHB, FFA, and PUN plasma

concentration using PROC Mixed in SAS 9.4.

Five covariance structures were tested for each outcome (simple, compound symmetry, autoregressive order 1, Toeplitz, and unstructured). Data from free AA concentration in plasma was unequally spaced therefore the covariance structures analyzed for each of the models were spatial power law, Gaussian and spherical. The covariance structure with the lowest Akaike's information criterion was selected. Fixed effects were treatment group and period using the REPEATED statement for the time variable. Block was included in every model as random effect. The treatment group × time interaction was forced in all models; other plausible interaction terms were tested, and not included in the final model if the *P*-value was ≥ 0.05. Tukey's post hoc test was used for multiple comparison correction of *P*-values for all pairwise comparisons of least square means. Normality and homoscedasticity of residuals was tested for each model fit. To meet the assumptions, the outcome variables BHB, FFA, free AA, and PUN concentrations were log transformed.

Results

Descriptive statistics of the study population by treatment group are presented in Table 4.3. The BCS is presented as median with range. Prepartum, BCS was 3.0 (3.0, 3.5) for the three groups. After calving, BCS for control, BCAA and BCAAPG was 3.0 (2.75, 3.25), 3.0 (2.75, 3.5) and 3.0 (2.75, 3.25).

Table 4.3. Descriptive statistics of the study population by treatment group; results are presented as total counts or average ± SD

	Treatment[1]			
Measurement	Control	BCAA	BCAAPG	*p*
Parity				
2	15	13	16	0.93
3	5	6	5	
≥4	6	4	4	
Days dry	58.6 ± 1.2	56.8 ± 1.3	56.9 ± 1.2	0.49
BW Dry	744.8 ± 13.9	717.8 ± 14.4	739.6 ± 14.0	0.27
BW Fresh	692.7 ± 12.2	697.5 ± 12.7	711.7 ± 12.3	0.43
Calving score				
1	16	16	19	0.43
2	8	6	3	
3	1	1	3	
4	1	0	0	
Calf weight (kg)	43.2 ± 1.2	41.2 ± 1.3	40.7 ± 1.2	0.31

[1] Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCAAPG) received 550 g/d of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

Milk yield and components

Milk yield, milk components, and ECM are shown in Table 4.4 and Figure 4.1. The MUN was higher for groups BCAA and BCAAPG than control (P = 0.01). The BCAA and BCAAPG supplementation had no effect on ECM (P = 0.11). The BCAA supplementation had no effect on any other milk components analyzed. Throughout the experiment, neither milk fat yield (P = 0.14) nor milk protein yield (P = 0.11) were affected by the treatment. An interaction between treatment group and period was detected for milk protein yield (P = 0.01). Protein yield for control period 1 was 3.30% (3.2, 3.4); control period 2, 3.71% (3.5, 3.93); BCAA period 1 3.53% (3.4, 3.7), BCAA period 2 3.69% (3.5, 3.9); for BCAAPG period 1 3.32% (3.2, 3.5); and, for BCAAPG period 2 3.76 (3.6, 4.0).

Table 4.4. Repeated measures ANOVA LSM (95% CI) for milk yield, energy corrected milk and milk composition

Variable	Treatments[1] (95% CI)			P-values for fixed effects[2]				
	Control	BCAA	BCAAPG	Tx	Time	P	Tx ×Time	Tx × P
Milk, Kg								
wk 1 to 5	41.7 (39.4, 44.0)	42.7 (40.3, 45.0)	43.7 (41.4, 46.0)	0.22	0.0001	0.29	0.06	-
wk 1	31.1 (28.4, 33.8)	30.7 (27.9, 33.5)	30.1 (27.4, 32.9)					
wk 3	44.4 (42.1, 46.7)	46.0 (43.7, 48.4)	47.9 (45.5, 50.2)					
wk 5	46.9 (44.4, 49.4)	48.5 (46.0, 51.0)	49.5 (47.0, 52.0)					
ECM, Kg								
wk 1 to 5	50.3 (46.8, 53.7)	52.4 (48.9, 55.8)	52.9 (49.5, 56.4)	0.11	0.0001	0.008	0.08	-
wk 1	45.0 (40.6, 49.4)	45.9 (41.4, 50.4)	42.8 (38.3, 47.2)					
wk 3	51.8 (48.4, 55.3)	54.6 (51.1, 58.0)	56.1 (52.5, 59.8)					
wk 5	51.4 (47.8, 54.9)	54.5 (50.9, 58.1)	55.4 (49.8, 59.0)					
Fat, %								
wk 1 to 5	4.58 (4.38, 4.80)	4.75 (4.53, 4.97)	4.65 (4.43, 4.97)	0.14	0.0001	0.07	0.28	-
wk 1	5.52 (5.17, 5.90)	5.76 (5.38, 6.17)	5.14 (4.80, 5.50)					
wk 3	4.36 (4.08, 4.66)	4.48 (4.18, 4.80)	4.46 (4.17, 4.78)					
wk 5	3.97 (3.72, 4.25)	4.26 (3.98, 4.57)	4.24 (3.96, 4.53)					
Protein, %								
wk 1 to 5	3.50 (3.33, 3.64)	3.61 (3.47, 3.75)	3.53 (3.39, 3.68)	0.11	0.0001	0.0001	0.89	0.02
wk 1	4.54 (4.30, 4.81)	4.86 (4.58, 5.15)	4.73 (4.46,5.01)					
wk 3	3.33 (3.15, 3.52)	3.46 (3.27, 3.67)	3.36 (3.17, 3.56)					
wk 5	3.06 (2.89, 3.24)	3.05 (2.88, 3.23)	3.00 (2.83, 3.18)					
MUN, %								
wk 1 to 5	8.60 (8.02, 9.22) [a]	9.70 (9.01, 10.45) [b]	9.75 (9.08, 10.47) [b]	0.01	0.28	0.003	0.28	-
wk 1	9.18 (8.15, 10.35)	9.73 (8.57, 11.04)	10.62 (9.40, 11.99)					
wk 3	8.18 (7.55, 9.20)	9.91 (9.10, 10.87)	9.55 (8.80, 10.36)					
wk 5	8.62 (7.81, 9.50)	9.33 (8.40, 10.35)	9.76 (8.83, 10.78)					

Table 4.4. (Continued)

Lactose, %								
wk 1 to 5	4.66 (4.59, 4.73)	4.67 (4.60, 4.74)	4.68 (4.60, 4.75)	0.94	0.0001	0.005	0.15	-
wk 1	4.40 (4.28, 4.51)	4.40 (4.29, 4.53)	4.49 (4.37, 4.61)					
wk 3	4.72 (4.65, 4.79)	4.76 (4.69, 4.84)	4.72 (4.65 4.79)					
wk 5	4.77 (4.69, 4.84)	4.74 (4.67, 4.82)	4.77 (4.69, 4.85)					
Total Solids, %								
wk 1 to 5	13.90 (13.51, 14.30)	14.16 (13.76, 14.56)	14.04 (13.65, 14.45)	0.24	0.0001	0.02	0.54	-
wk 1	15.71 (15.11, 16.33)	16.26 (15.63, 16.93)	15.69 (15.08, 16.31)					
wk 3	13.50 (13.11, 13.91)	13.77 (13.36, 14.19)	13.64 (13.24, 14.06)					
wk 5	12.87 (12.47, 13.29)	13.09 (12.68, 13.53)	13.11 (12.07, 13.55)					
Linear Scores[3],								
wk 1 to 5	0.96 (0.73, 1.26)	1.00 (0.75, 1.32)	1.09 (0.83, 143)	0.80	0.0001	0.19	0.74	-
wk 1	2.62 (2.13, 3.22)	2.77 (2.22, 3.45)	2.59 (2.09, 3.21)					
wk 3	0.70 (0.49, 1.01)	0.78 (0.54, 1.14)	0.97 (0.67, 1.41)					
wk 5	0.53 (0.31, 0.90)	0.42 (0.25, 0.71)	0.55 (0.33, 0.92)					

[1]Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCAAPG) received 550 g/d of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

[2]Fixed effects were treatment group (Tx), time, period (P) and 2-way interactions; interactions with a P-value ≥ 0.05 were excluded from the model with the exception of Tx ×Time.

[3]Linear scores (LS) were calculated as: LS = [ln (SCC/100)/ln(2)]+3. Detailed LSM of Group × Period interaction included in the main text

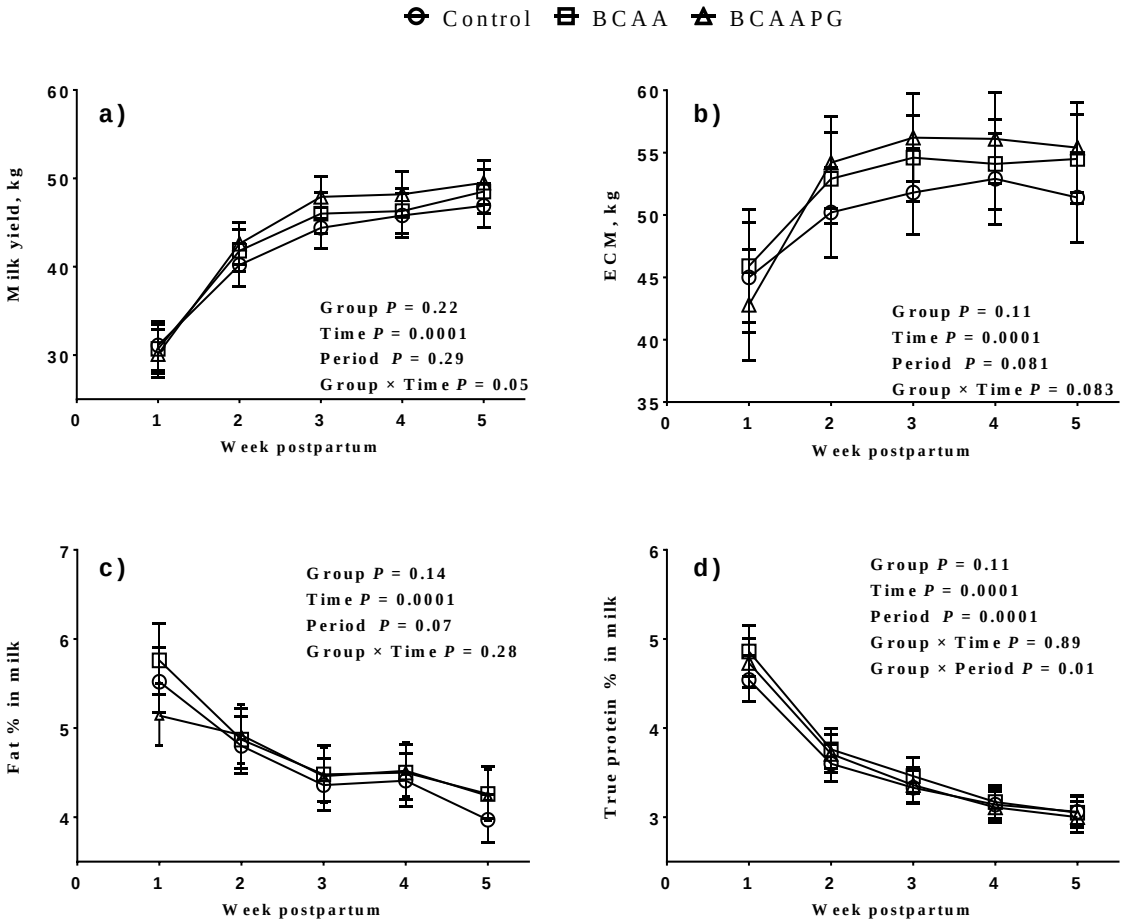

Figure 4.1. Least square means and 95% CI for ANOVA of milk yield (a), ECM (b), fat % in milk (c) and, true protein % (d). Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCAAPG) received 550 g/d of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM. Fixed effects were treatment group (Tx), time, period (P) and 2-way interactions; interactions with a P-value ≥ 0.05 were excluded from the model with the exception of Tx ×Time.

Diet analysis

The composition analysis of prepartum and postpartum diet is presented in Table 4.5. The DMI throughout prepartum period for control, BCAA and BCAAPG is shown in Figure 4.2. Postpartum, the DMI was not different among the control, BCAA and BCAAPG groups (P = 0.13). The BW lost (95%CI) from 1 wk until 6 wk postpartum was 11.0 (-7.0, 29.0), 23.6 (4.8, 42.3), and 17.8 (-0.4, 35.9) for control, BCAA and BCAAPG, in that order (P = 0.54). Free BCAA concentrations in plasma during pre and postpartum periods are shown in Figure 4.3. The concentration in plasma of the three BCAA did not differ among the groups. Postpartum, L-Leu average concentration in plasma was 80.05, 88.34, and 85.81 μM for control, BCAA and BCAAPG, respectively (group P = 0.07). L-Iso concentration in plasma postpartum was 61.24, 66.72, and 62.41 μM for control, BCAA and BCAAPG, in that order (group P = 0.36). L-Val concentration in plasma postpartum was 136.81, 152.91, and 147.28 μM for control, BCAA and BCAAPG, correspondingly (Group P = 0.04).

Model predicted EB and PB pre- and postpartum are shown in Table 4.6 and Figure 2. The predicted energy and protein density for the dry period diet were 2.11 Mcal of ME/kg and 99.1 g of MP/kg. The estimated duodenal flows of digestible EAA (CNCPS v. 6.5) are presented in Table 4.7.

Blood metabolites

The BHB, FFA and PUN concentration in plasma are presented in Table 4.8. Plasma urea nitrogen (95% CI) was different during the dry period among the control, BCAA and BCAAPG groups (P = 0.02) as well as postpartum (P = 0.001). Postpartum,

FFA (95% CI), concentration in plasma was not affected by the supplementation with BCAA or BCAAPG (P = 0.6). Plasma BHB during prepartum was similar among all groups (P = 0.29). The concentrations of plasma BHB from calving until 21 DIM was not altered by supplementation with BCAA or PG administration (P = 0.35). The number of plasma samples classified as HYK from calving until 21 DIM was 77/241 (11.3%), 44/213 (6.5%) and 57/228 (8.4 %) in groups control, BCAA and BCAAPG, respectively (P = 0.02).

Table 4.5. Average (SD) chemical composition of the diets.

Component[1]	Dry	Fresh
DM %	46.7 ± 4.6	48.5 ± 3.18
NE_L Mcal/kg DM	1.48 ± 0.02	1.63 ± 0.01
CP % DM	13.6 ± 0.9	15.5 ± 0.8
Soluble protein	5.4 ± 0.5	6.65 ± 0.4
ADF % DM	28.2 ± 2.3	21.6 ± 1.6
aNDF % DM	42.2 ± 3.3	31.7 ± 2.1
Starch % DM	19.5 ± 3.1	25.7 ± 2.7
Ether extract % DM	3.1± 0.3	3.4 ± 0.2
Ash % DM	8.2 ± 0.7	7.9 ± 0.5
Ca % DM	1.54 ± 0.35	0.89 ± 0.09
P % DM	0.31 ± 0.04	0.36 ± 0.03
Mg % DM	0.52 ± 0.07	0.46 ± 0.05
K % DM	1.20 ± 0.16	1.28 ± 0.41
S % DM	0.42 ± 0.05	0.37 ± 0.03
Na % DM	0.13 ± 0.02	0.77 ± 0.11
Cl % DM	0.64 ± 0.07	0.51 ± 0.05
DCAD meq/100g DM	-8.05 ± 5.5	31.0.2 ± 5.25

[1]Values represent averages of weekly samples composited and do not include top-dress supplement or the oral drench. Chemical composition is the average ± SD of a weekly sampling analyzed by near infrared reflectance spectroscopy (NIR; Cumberland Valley Analytical Services, Waynesboro, PA).

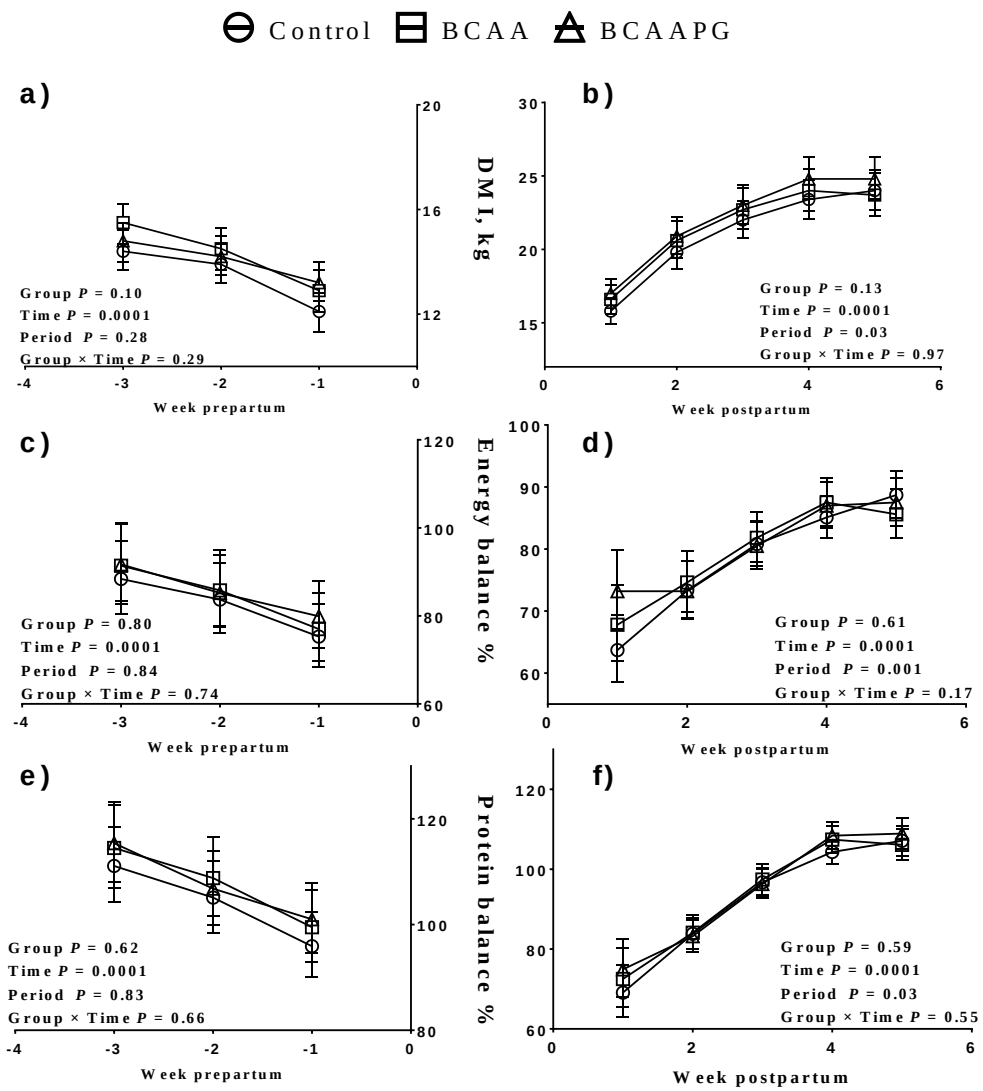

Figure 4.2. Dry matter intake (Kg) wk 3 to 1 prepartum (a) and wk 1 to 5 postpartum (b) for the 3 treatments groups. Energy balance during the prepartum period (c) and postpartum period (d) as percentage of the requirements. . Protein balance during the prepartum period (e) and postpartum period (f) as percentage of the requirements. Data is presented as least square means and 95% CI. Energy and protein balance was calculated with Cornell Net Carbohydrate and Protein System (CNCPS v. 6.5) for each cow during dry and fresh periods using a weekly average of BW, DMI and either days carried calf or milk yield and milk components. Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCAAPG) received 550 g/d of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

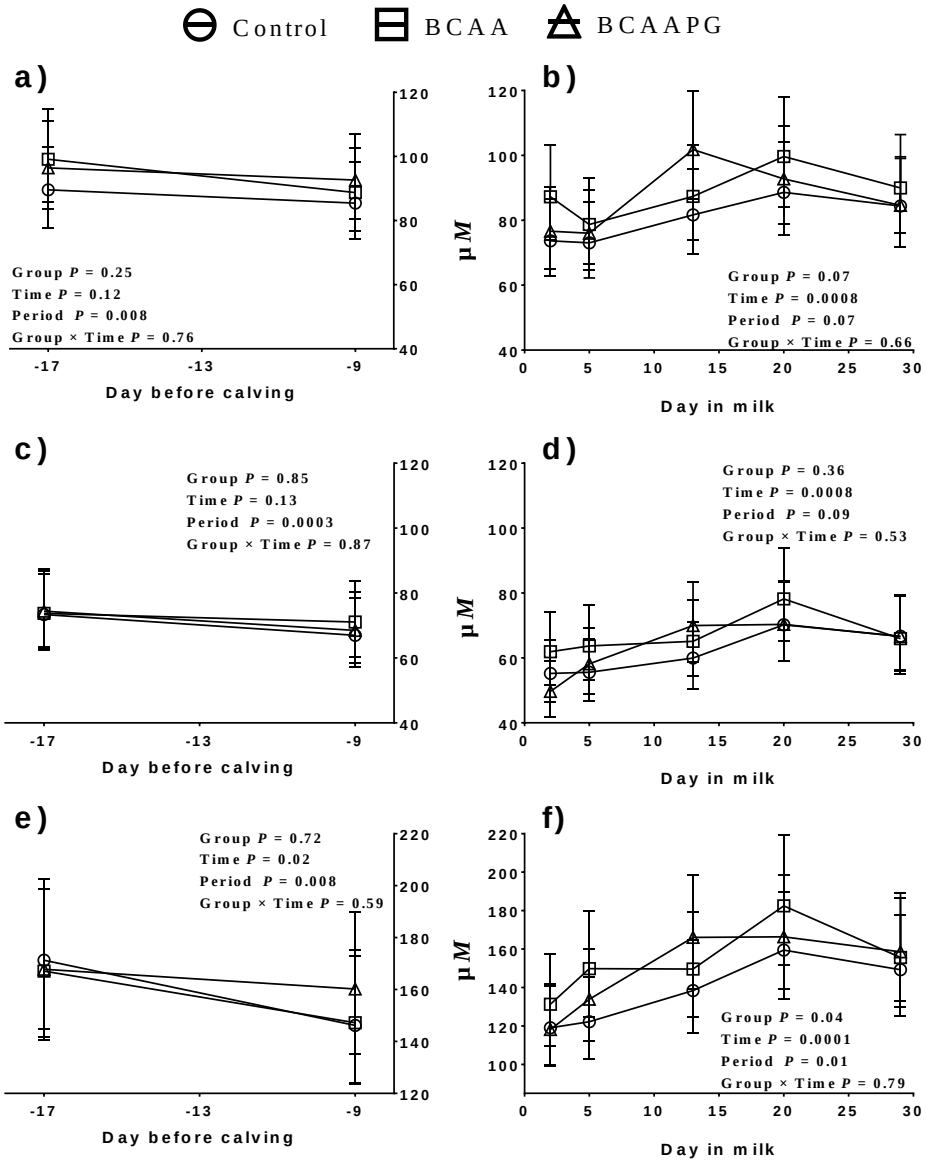

Figure 4.3. Least square means and 95% CI for ANOVA of a) free L-Leu concentration in plasma prepartum; b) free L-Leu concentration in plasma postpartum; c) free L-Iso concentration in plasma prepartum; d) free L-Iso concentration in plasma postpartum; e) free L-Val concentration in plasma prepartum and; f) free L-Val concentration in plasma postpartum. Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCAAPG) received 550 g/d of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

Table 4.6. Repeated measures ANOVA LSM (95% CI) for energy and protein balance estimates calculated with the Cornell Net Carbohydrate and Protein Model (CNCPS 6.5)

Variable	Treatments (95%CI)			P-values for fixed effects[2]				
	Control	BCAA	BCAAPG	Tx	Time	P	Tx × Time	Tx × P
EB, % Dry								
wk -3 to -1	82.3 (75.4, 89.7)	84.5 (77.1, 92.7)	85.5 (78.2, 93.4)	0.80	0.0001	0.84	0.74	-
wk -3	88.4 (80.5, 97.1)	91.4 (82.7, 101.0)	91.7 (83.3, 100.9)					
wk -1	75.3 (68.5, 82.7)	77.0 (69.7, 85.1)	79.9 (72.6, 88.0)					
PB, % Dry								
wk -3 to -1	103.8 (97.9, 110.1)	107.4 (101.0, 114.3)	107.5 (101.3, 114.2)	0.62	0.0001	0.83	0.66	-
wk -3	111.1 (104.2, 118.4)	114.5 (107.0, 122.6)	115.4 (108.1, 123.2)					
wk -1	95.9 (90.0, 102.3)	99.5 (93.0, 106.5)	101.0 (94.6, 107.8)					
EB, % Fresh								
wk 1 to 5	77.8 (74.6, 81.0)	79.1 (75.7, 82.6)	80.0 (76.7, 83.5)	0.61	0.0001	0.001	0.17	-
wk 1	63.5 (58.5, 69.3)	67.8 (61.9, 74.2)	73.2 (67.1, 79.9)					
wk 2	73.3 (68.8, 78.1)	74.6 (68.7, 78.1)	73.2 (68.7, 78.1)					
wk 5	88.7 (85.0, 92.6)	85.6 (81.8, 89.6)	87.5 (83.7, 91.4)					

Table 4.6. (Continued)

PB, % Fresh								
wk 1 to 5	91.1 (87.9, 94.3)	92.5 (89.1, 96.0)	93.3 (90.0, 96.8)	0.59	0.0001	0.03	0.55	-
wk 1	69.1 (62.9, 76.0)	72.5 (65.5, 80.2)	74.9 (68.0, 82.5)					
wk 2	83.8 (79.9, 87.8)	84.1 (80.0, 88.5)	83.2 (79.3, 87.3)					
wk 5	107.1 (103.4, 110.9)	106.1 (102.2, 110.1)	108.9 (105.1, 112.9)					

[1] Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCAAPG) received 550 g/d of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

[2]Fixed effects were treatment group (Tx), time, period (P) and 2-way interactions; interactions with a P-value ≥ 0.05 were excluded from the model with the exception of Tx ×Time.

Table 4.7. Predicted diet composition

	Treatments[1]			
	Control	BCAA	BCAAPG ≤ 7 DIM	BCAAPG > 7 DIM
Predicted Composition[2]				
ME (Mcal/Kg of DM)	2.53	2.50	2.50	2.49
MP (g/kg of DM)	120.6	121.8	122.8	123.8
MP (g/d)	2955	3083	3117	3083
Methionine	90.8	96.9	99.9	98.6
Lysine	236.1	252.0	260.1	256.5
Arginine	182.0	194.3	200.7	197.8
Threonine	146.0	155.8	160.9	158.0
Leucine	258.1	284.9	293.7	290.0
Isoleucine	143.5	158.6	164.0	161.4
Valine	183.0	206.7	213.2	210.4
Histidine	90.3	96.5	99.4	98.2
Phenylalanine	161.0	171.9	177.3	175.0
Tryptophan	46.0	49.0	50.7	49.9

[1] Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCAAPG) received 550 g/d of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

[2]Predicted by Cornell Net Carbohydrate and Protein System (v 6.55, Cornell University, Ithaca, NY) based on composite forage analysis, average ingredient composition, and with observed mean DMI, BW, milk yield and components for each treatment group.

Table 4.8. Repeated measures ANOVA LSM for β-hydroxybutyrate (BHB), free fatty acids (FFA) and plasma urea nitrogen (PUN) during dry and fresh period

Variable	Treatments[1] (95% CI)			P-values for fixed effects[2]				
	Control	BCAA	BCAAPG	Tx	Time	P	Tx ×Time	Tx × P
BHB (mmol/L), Dry								
Day -18 to -2	0.6 (0.5, 0.6)	0.5 (0.5, 0.6)	0.5 (0.5, 0.6)	0.29	0.0001	0.33	0.07	-
d -18	0.5 (0.5, 0.5)	0.5 (0.4, 0.5)	0.5 (0.5, 0.6)					
d -12	0.5 (0.5, 0.6)	0.5 (0.5, 0.6)	0.5 (0.5, 0.5)					
d -8	0.6 (0.5, 0.6)	0.5 (0.5, 0.6)	0.5 0.5, 0.6)					
d -2	0.7 (0.6, 0.8)	0.6 0.5, 0.7)	0.5 (0.5, 0.6)					
FFA (µeq/L), Dry								
Day -18 to -2	197.7 (173.3, 225.5)	163.0 (141.8, 187.2)	168.0 (146.8, 192.2)	0.06	0.0001	0.86	0.04	-
d -18	132.1 (109.5, 159.4)	120.1 (99.3, 145.2)	114.1 (92.4, 141.0)					
d -12	166.6 (140.8, 197.2)	123.6 (103.1, 148.3)	130.0 (109.9, 153.8)					
d -8	212.6 (179.9, 251.2)	177.9 (148.7, 212.9)	182.7 (154.2, 216.4)					
d -2	383.9 (324.0, 454.9)	277.3 (231.4, 332.5)	289.5 (243.3, 344.5)					
PUN (mg/dL), Dry								
Day -18 to -2	13.6 (13.3, 15.0)[a]	15.0 (14.0, 16.0)[b]	14.2 (13.3, 15.2)[ab]	0.02	0.19	0.89	0.17	0.03
d -18	14.1 (12.9, 15.4)	14.9 (13.6, 16.4)	15.5 (13.9, 17.2)					
d -12	13.7 (12.6, 14.8)	14.8 (13.5, 16.1)	13.6 (12.5, 14.7)					
d -8	13.2 (12.1, 14.3)	14.8 (12.1, 14.3)	14.2 (13.1, 15.5)					
d -2	13.7 12.6, 14.9)	15.0 (13.7, 16.4)	13.7 (12.6, 14.9)					
BHB (mmol/L), Fresh								
DIM 1 to 21	0.8 (0.7, 1.0)	0.7 (0.6, 0.9)	0.8 (0.6, 0.9)	0.35	0.02	0.90	0.35	-
d 1	0.8 (0.6, 1.0)	0.7 (0.5, 0.9)	0.6 (0.5, 0.8)					
d 5	0.9 (0.7, 1.2)	0.7 (0.6, 1.0)	0.7 (0.6, 0.9)					
d 11	0.8 (0.6, 1.1)	0.6 (0.5, 0.8)	0.8 (0.7, 1.1)					
d 15	0.8 (0.6, 1.1)	0.7 (0.5, 0.9)	0.8 (0.6, 1.1)					
d 21	0.9 (0.7, 1.2)	0.9 (0.7, 1.2)	0.9 (0.7, 1.1)					

Table 4.8. (Continued)

FFA (µeq/L), Fresh								
DIM 1 to 21	581.4 (503, 671.9)	524 (450.4, 611.8)	545.4 (470.3, 632.3)	0.61	0.0001	0.84	0.27	-
d 1	623.3 (513.8, 756.0)	468.2 (378.2, 579.6)	546.5 (484.7, 616.2)					
d 5	676.4 (553.4, 827.0)	642.8 (518.6, 796.9)	596.8 (529.1, 673.1)					
d 11	620.6 (508.7, 756.8)	600.3 (484.4, 744.0)	618.6 (548.7, 697.4)					
d 15	473.8 (389.9, 575.8)	471.5 (381.4, 582.8)	501.2 (444.9, 564.7)					
d 21	448.0 (356.0, 564.0)	454.4 (363.0, 568.7)	448.1 (394.0, 516.9)					
PUN (mg/dL), Fresh								
DIM 1 to 21	8.3 (7.7, 8.9)	10.1 (9.4, 10.9)	9.6 (9.4, 10.3)	0.001	0.0001	0.38	0.57	-
d 1	9.6 (8.7, 10.6)	11.7 (10.5, 13.0)	10.4 (9.3, 11.5)					
d 5	8.6 (7.6, 9.3)	10.0 (9.0, 11.2,)	9.7 (8.1, 10.7)					
d 11	8.4 (7.6, 9.3)	9.7 (8.7, 10.8)	9.6 (8.6, 10.6)					
d 15	7.8 (7.0, 8.6)	9.8 (8.8, 10.9)	9.6 (8.7, 10.7)					
d 21	8.3 (7.4, 9.3)	9.4 (8.4, 10.5)	9.8 (8.9, 10.9)					

[1]Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCAAPG) received 550 g/d of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

[2]Fixed effects were treatment group (Tx), time, period (P) and 2-way interactions; interactions with a P-value ≥ 0.05 were excluded from the model with the exception of Tx ×Time.

Discussion

The overall goal of our study was to test the effect of supplementation with BCAA on milk yield, milk components and blood metabolites during the early postpartum period in dairy cows. Additionally, we wanted to test the effect of PG as glucose precursor enhancing the response of BCAA by increasing energy status and circulating insulin, thereby sparing AA for physiological functions other than energy production.

The BCAA supplementation with or without oral PG did not affect DMI, but DMI increased as lactation advanced during the first 5 wk postpartum. The lack of treatment effect on DMI is in agreement with previous studies using BCAA or casein infusion during early lactation (Larsen et al., 2014) or mid to late lactation (Mackle et al., 1999a, Nichols et al., 2016, Curtis et al., 2018). All EAA have a gluconeogenic potential in ruminants as well as being a milk protein precursor (Bauman and Currie, 1980, Overton et al., 1999, Larsen and Kristensen, 2013).

The EB postpartum was not affected by BCAA or BCAAPG supplementation. The CP and MP across the two treatments and the control group were essentially identical during the postpartum period. The PB postpartum was not different between control, BCAA and BCAAPG groups. Protein supplementation had a significant effect on DMI and milk protein yield when the diets are deficient in MP (Allen, 2000), but the response is reduced if the diets are meeting or exceeding the protein requirements (Martineau et al., 2016).

In our study, the CNCPS 6.55 predicted an increase on BCAA duodenal flow in the treatments groups compared with the control group. The prediction of EAA

duodenal flow is a good indicator of free plasma EAA in dairy cows (Pacheco et al., 2012, Patton et al., 2015). Sadri et al. (2017) reported an increase in plasma concentration of Leu after a duodenal bolus infusion. On the other hand, jugular infusion of BCAA had no effect on plasma concentration of BCAA (Kassube et al., 2017). Changes in concentration of plasma free BCAA could be due to reduced DMI, BCAA absorption in the intestine or enhanced uptake for catabolism or protein synthesis. For our study, L-Leu dose differences in the first 10 enrollment blocks of the study might affect free BCAA concentration in plasma.

Even though milk yield and ECM were not statistically altered by BCAA or BCAAPG supplementation during early lactation, mean milk yield and mean ECM were lower in the control group than the groups supplemented with BCAA or BCAAPG. Milk yield response to BCAA or casein infusion has been reported before, ranging from no or small response (Broderick et al., 1970, Appuhamy et al., 2011, Doelman et al., 2015) to a large increase (McCormick et al., 1999, Larsen et al., 2014, Martineau et al., 2017). Milk production differences among the studies could be caused by different factors including the dose of BCAA or other EAA infused, stage of lactation at time of treatment and, other limiting nutrients in the diets (Larsen et al., 2014). An increase in percentage of milk protein has been observed after AA supplementation in dairy cows (Rulquin et al., 2006, Appuhamy et al., 2011), but BCAA supplementation did not alter milk composition in our study. Mammary gland uptake of EAA is in excess of what is necessary for protein synthesis (Bequette et al., 1998). The partitioning of EAA in the body changes during anabolic and catabolic stages (Bequette et al., 1997) and, oxidation of AA in splanchnic, muscle and other tissues may be a limiting factor for protein

synthesis (Reynolds et al., 1994). Differences in milk protein yield between the two study periods were similar among all groups and may be due to season or feedstuff variability but are likely not exclusively due to the differences in L-Leu dose as they affected all groups equally. In the present study, MUN and PUN concentrations were increased with BCAA supplementation similar to previous studies infusing BCAA (Nichols et al., 2016) and casein (Martineau et al., 2017). High concentration of MUN and PUN point out an overall surplus of nitrogen in the cow and might be caused by several factors including energy-protein imbalance in the diet (Kohn, 2007), the dietary concentration of CP and metabolism of RDP and RUP pulls (Roseler et al., 1993, Broderick and Clayton, 1997, Huhtanen et al., 2015). Results did not differ between BCAA and BCAAPG, suggesting that added gluconeogenic precursors in the form of PG did not affect a possible energy-protein imbalance postpartum. Before calving, the control group had the lowest DMI among all groups, this might impact the PUN concentration during the dry period. Postpartum, the DMI and CP among control, BCAA and BCAAPG groups were similar, therefore the differences in PUN and MUN concentration among the groups are likely due to the BCAA supplement.

Mammary gland uptake of BCAA from blood is often in excess of what is required for typical milk protein yield (Wohlt et al., 1977, Lei et al., 2012). When inside the cell, free BCAA may be synthesized to form milk proteins, preserved within the cell for structural proteins synthesis, used as precursor for different metabolic and catabolic processes, or passed unaltered into milk, blood, or lymph (Mepham, 1982, Meijer et al., 1995, Larsen et al., 2015). The variety of metabolic pathways for free BCAA might explain the lack of response in milk components in our study or our dose was not

sufficient to alter these parameters.

Predicted AA flow by CNCPS modeling shows an increased flow of BCAA in the two treatments compared with the control group. Also, supplementation with BCAA as a top dress from calving until 35 DIM produced a significant increase in free Val concentration in plasma and moderate increase in free Leu and Iso plasma concentrations. Kassube et al (2017) did not find an increase in concentrations of free plasma BCAA after intravenous infusion of a mix of methionine, lysine and BCAA. In our study, dose and time of sampling might play a role in being able to detect the moderate increase in free Leu and Val concentration in plasma.

The prophylactic administration of PG was not associated with any improvements in productive or reproductive performance in dairy cows (Hoedemaker et al., 2004, Chung et al., 2009a), but PG was effective for reducing blood metabolites associated with lipolysis such as FFA and BHB (Pickett et al., 2003, McArt et al., 2012). Despite the positive effects of PG as oral drench in hyperketonemic cows (McArt et al., 2011, Gordon et al., 2017), the use of PG as a blanket therapy has shown discrepant results. Some studies have shown positive effects such as reducing FFA and BHB concentrations, while increasing insulin concentrations in blood when different doses of PG were administered as oral drench as a blanket therapy (Chung et al., 2009b, Maurer et al., 2017). Other studies have shown no effect on milk yield, milk components, EB, BHB, or FFA concentrations in blood when liquid or dry PG was offered as a top dress or mixed into the TMR (Chibisa et al., 2008, Chung et al., 2009a, Lomander et al., 2012). However, there is precedent of unaffected BHB concentrations after oral drench 300ml of PG as a blanket therapy in dairy cows (Formigoni et al., 1996). In our study,

the BCAA group had significantly fewer plasma samples classified as HYK compared to the control and BCAAPG groups. Supplementation with BCAA in other species improves glucose metabolism in skeletal muscle, adipose tissue and liver (Takumi et al., 2011). Isoleucine has been shown to increase FFA oxidation in skeletal muscle and liver in mice (Nishimura et al., 2010) and, Leu is likely a nutrient signal that can affect DMI as well as EB (Lynch and Adams, 2014, Yoon, 2016).

Numerous elements have the potential to alter free BCAA blood concentration. Insulin and glucose have been reported to modify plasma BCAA concentrations (Felig et al., 1969, Layman et al., 2003, Shin et al., 2014). Catabolism of BCAA produces substrates to maintain active essential pathways for the liver mitochondria via TCA cycle (Sunny et al., 2015). Insulin and free BCAA blood concentrations regulate protein turnover within the organism. Supplementation with BCAA probably can both increase substrate supply and activate regulatory protein factors (Vary and Lynch, 2007, Lynch and Adams, 2014). Theoretically the use of PG, a glucose precursor and insulin stimulant in combination with BCAA, during catabolic stages might potentiate the nutraceutical effects of BCAA in dairy cows.

When interpreting the results of our study, it has to be acknowledged that we lacked a treatment group with only PG supplementation and therefore are unable to assess the effect caused by PG alone. Despite this, few differences between the BCAA and BCAAPG group were noted, making it unlikely that PG alone was responsible for observed differences in comparison with the control group. The dose of BCAA used during the present study was formulated based on previous studies using post-ruminal infusion of BCAA and casein in mid and early lactation in dairy cows. The quantities

needed to produce a measurable impact on production using BCAA supplementation during early lactation require more evaluation.

Conclusion

We presented the effects of BCAA with or without PG supplementation during early lactation in dairy cows on different productive parameters and concentration of energy metabolites. Diets with high concentrations of BCAA have shown beneficial effect on body weight, glucose concentrations in blood, and increased muscle synthesis in monogastrics during inflammation and catabolic processes. We conclude that BCAA supplementation in this study did not have an effect on DMI, milk yield and milk protein. However, BCAA increased PUN and MUN as well as free Val concentration in plasma. BCAA alone reduced the number of plasma samples classified as HYK during the first 21 DIM. For this reason, the use of BCAA as supplement for dairy cattle deserves further investigation.

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CHAPTER 5

POSTPARTUM SUPPLEMENTATION WITH RUMEN PROTECTED BRANCHED-CHAIN AMINO ACIDS: EFFECT ON LIVER FUNCTION

Abstract

Essential amino acids (**EAA**) are critical for multiple physiological processes. Branched-chain amino acid (**BCAA**) supplementation has a positive effect on body weight, fat tissue and insulin resistance in several species. The objective was to evaluate the effect of rumen protected branched-chain amino acids (**RP-BCAA;** 375 g of 27% L-Leucine, 85 g of 48 % L-Isoleucine and 91 g of 67% L-Valine) with or without propylene glycol (**PG**) oral administration on liver function, free fatty acids (**NEFA**), β-hydroxybutyrate (**BHB**), and liver triglycerides (**TG**) concentration during the early postpartum period in dairy cows. Multiparous Holsteins were enrolled in blocks of three and randomly assigned to either the control group or one of the two treatments from calving until 35 d. The **Control** group (n=16) received 200g of dry molasses; the **RP-BCAA** group (n=14) received RP-BCAA mixed with 200g of dry molasses; the RP-BCAA plus PG (**RP-BCAAPG**) group (n=16) received RP-BCAA mixed with 200g of dry molasses plus 300 ml of PG once daily from calving until 7 DIM. Liver biopsies were collected at day 9 ± 4 pre-partum and day 5 ± 1, and day 20 ±1 post-partum. Blood was sampled three times per week from calving until 21 DIM. Milk yield, dry matter intake (**DMI**), NEFA, BHB, liver enzymes, and TG were analyzed using repeated measurements ANOVA. In monogastrics, BCAA supplementation upregulates the hepatic sterol regulatory element-binding protein/ liver x receptor pathway and key

factors in cholesterol and NEFA metabolism. Also, BCAA supplementation may increase oxidation of NEFA via peroxisome proliferator-activated receptor α. The combination of BCAA and PG likely provides substrates for gluconeogenesis, reduces mobilization of NEFA, and may enhance NEFA utilization within the hepatocyte. Therefore, the use of RP-BCAA in combination with PG might be a feasible option to reduce hepatic lipidosis in dairy cows during early lactation.

Introduction

The liver is a crucial regulator of metabolic pathways within the organism. The time between feeding and reaching the fasted stage is characterized by a change in substrate supply for the liver from mainly glucose utilization and fatty acid synthesis to fatty acid oxidation (McGarry and Foster, 1980). The dry matter intake (**DMI**) is reduced during early postpartum and is insufficient to supply nutrients for the increasing milk synthesis and to support maintenance (Allen, 2000, Ji and Dann, 2013, Van Saun and Sniffen, 2014). The nutrient shortage is compensated by mobilizing body reserves (Bauman and Currie, 1980, Bell et al., 2000, Mann et al., 2016a). The major energy storage in the body is the adipose tissue. During the shortage of nutrients, adipose tissue is mobilized into the blood stream as free fatty acids (**FFA**). During early postpartum, the liver experiences an elevated influx of FFA, surpassing the oxidation and re-esterification rates of FFA in the hepatocyte (Grummer, 1993, Newman et al., 2016). The surplus of FFA are stored as triglycerides (**TG**) and is associated with declines in milk yield and fertility in dairy cows (Jorritsma et al., 2000, Bobe et al., 2004). Excessive accumulation of TG stresses the hepatocyte and stimulates cellular apoptotic signaling (Unger and Orci, 2002, Malhi et al., 2006)

The liver also increases uptake of free amino acids (**AA**) from the blood stream during early postpartum and utilizes them as either an energy substrate or for protein synthesis. Hepatic lipidosis impairs the normal metabolic functions within the hepatocyte and may affect the plasma essential amino acids (**EAA**) concentration (Shibano and Kawamura, 2006).

The branched-chain amino acids (**BCAA**; isoleucine, leucine and valine) are

three of the known EAA in dairy cows. Therefore, BCAA daily consumption is required to maintain their concentration in a normal range within the organism. Plasma BCAA concentration in dairy cows starts decreasing before calving and reaches a nadir on day 1 after calving, returning to normal levels approximately two weeks postpartum (Kuhla et al., 2011, Zhou et al., 2016). BCAA supplementation regulates glucose homeostasis and protein metabolism in several species (Shimomura et al., 2006, Torres-Leal et al., 2011, Lynch and Adams, 2014), stimulating the mammalian target of rapamycin (mTORC) pathway and inducing increased protein synthesis (Kimball and Jefferson, 2006, Lynch and Adams, 2014, Yoon, 2016).

Dietary BCAA supplementation has reduced hepatic TG accumulation in rodents fed high fat diets (Arakawa et al., 2011), improves liver function in humans and rodents with non-alcoholic hepatic lipidosis disease (**NAFLD**) (Marchesini et al., 2005) and reduces mRNA of some lipogenic genes in the liver of female broilers (Bai et al., 2015).

Energy and protein needs are strongly connected within the organism. Propylene glycol (**PG**) has been used widely in dairy cows for treatment of hyperketonemia (**HYK**). Shortly after oral administration, PG is fermented and absorbed as propionate from the rumen then used by the liver as glucose precursor. The PG oral administration also triggers metabolic pathways to maintain hemostasis within the organism, such as increased insulin secretion and decreased concentrations of FFA and β-hydroxybutyrate (**BHB**) (Grummer, 1993, Christensen et al., 1997, Nielsen and Ingvartsen, 2004). Total liver lipids and hepatic TG were reduced during early postpartum in cows treated with PG (Studer et al., 1993, Pickett et al., 2003).

Therefore, our hypothesis is that rumen protected branched chain amino acids (**RP-BCAA**) alone or in combination with PG will ameliorate liver dysfunction caused by excessive FFA mobilization during early lactation in Holstein dairy cows. The objective of our study was to evaluate the effect of BCAA with or without PG oral administration on liver metabolism and signaling during the early postpartum period in Holstein dairy cows.

Materials and methods

Study population, diets and treatments

All procedures were approved by the Cornell University Institutional Animal Care and Use Committee (protocol # 2011-0016). In depth description of the study population, diets, feed chemical composition, energy and protein balance was described previously (Table 4.2 and Table 4.5). Briefly, cows entering second or greater lactation were randomly enrolled into one of the following groups: control (n=17; 200 g/d of dry molasses only from calving to 35 DIM); BCAA (n=17; 550 g/d of BCAA with 200g of dry molasses from calving to 35 DIM); and BCCAPG (n=17; 550 g/d of BCAA with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM). Energy and protein balance were estimated with the Cornell Net Carbohydrate and Protein System software (CNCPS; Cornell University, version 6.5).

Animals for this study were selected from a larger study population based on the dose of L-Leu received during the study period. The BCAA was composed of 375 g (27 % wt./wt.) rumen protected L-Leu, 85 g (48 % wt./wt.) rumen protected L-Ile and 91 g of (67 % wt./wt.) rumen protected L-Val.

Sample collection

Cows were enrolled 28 days before expected calving date and body weight (**BW**) was determined after the morning milking and body condition score (**BCS**), on a 1-5 scale (Edmonson et al., 1989), was recorded on a weekly basis. Milk yield was recorded daily and milk samples were collected from three consecutive milkings once a week and analyzed for components later used to calculate energy corrected milk (Mann et al., 2015).

Blood was sampled 3 times per wk 21 days before expected calving until 21 DIM from the coccygeal vessels using 20-gauge x 2.54 cm needles and blood collection tubes (Becton, Dickinson and Company Becton Drive Franklin Lakes, New Jersey) containing 158 USP of sodium heparin and without anticoagulant, for plasma and serum separation. All blood samples were immediately placed on ice and plasma and serum were separated within 1 h at 2,800 × *g* for 20 min at 4°C then stored at -20° until analysis.

Glucose (PGO enzyme preparation, Sigma Aldrich, St. Louis, MO) and FFA (HR Series NEFA-HR (2); Wako Life Sciences, Mountain View, CA) concentrations in plasma were estimated by colorimetric measurement of an enzymatic reaction (Mann et al., 2015). The BHB-Check Ketone meter system (Pharma DOC, Lübeck, Germany) was used to measure plasma BHB concentration (Leal Yepes et al., 2018). HYK was defined as BHB ≥ 1.2 mmol/L (Ospina et al., 2013). Radioimmunoassays were used to measure plasma insulin (Insulin RIA kit, RI-13 K, EMD Millipore, St. Charles, MO) and glucagon (Glucagon RIA kit, GL-32 K, EMD Millipore) for duplicate samples in the Endocrinology Laboratory of the New York State Animal Health Diagnostic Center

(Mann et al., 2016b). Molar insulin:glucagon ratio was calculated as previously described (Mann et al., 2016b).

Plasma free AA concentrations were established using EZ:faast (Phenomenex, Torrance, USA) by the Chromatography and Diagnostic Services Laboratory of the Veterinary Faculty, University of Montréal as previously described (Kassube et al., 2017).

Serum samples on the day of the biopsies were analyzed for total protein, albumin, globulin, aspartate transaminase (**AST**), sorbitol dehydrogenase (**SDH**); glutamate dehydrogenase (**GLDH**), gamma-glutamyl transferase (**GGT**), alanine aminotransferase (**ALT**), alkaline phosphatase, and creatine kinase at the Clinical Pathology Laboratory of the New York State Animal Health Diagnostic Center using an automated wet chemistry analyzer (Cobas c501, Roche Diagnostics, Indianapolis, IN).

Liver biopsy

Liver biopsies were obtained via percutaneous trocar approach (Hughes, 1962, Veenhuizen et al., 1991). Hair was clipped from the 11th intercostal space then washed with iodine soap and dried with paper towels. Mild sedation with acepromazine maleate (0.003 to 0.005 mg/kg, IV, VetOne) was provided. The biopsy site in the right 11th intercostal space was chosen after confirmation of correct placement with ultrasonography using a 7.5-MHz linear-array transducer (Ibex Pro, E.I. Medical Imaging, Loveland, CO) and 70% ethanol (Vet One, Boise, ID). The area was surgically prepared then local anesthesia was accomplished with 10 mL of a 2% lidocaine solution (lidocaine 2% HCl; Vet One). A skin incision at the 11th intercostal space was made

using a #22 scalpel blade to place the stainless-steel trocar into the abdominal cavity, directing the point of the trocar toward the left elbow. Tissue samples were placed on a 7.62- × 7.62-cm sterile nonwoven sponge to remove excess blood and immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis. The incision was closed with a disposable skin stapler (3M Precise, St. Paul, MN) then coated with aluminum spray bandage. All cows received flunixin meglumine (1.1 mg/kg, IV Prevail, VetOne) for pain control immediately after the biopsy.

Protein extraction and immunoblotting

The protein extraction and immunoblotting procedure was previously reported (Mann et al., 2016a). Briefly, around 50 mg frozen liver tissue was homogenized, transferred to 1 mL of ice-cold lysis buffer containing 25 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA and 1 % Triton X-100 and 1 mM PMSF with the addition of a phosphatase and protease inhibitor cocktail (Halt, Thermo Fisher Scientific, Waltham, PA), placed on ice for 20 min then centrifuged at 10,000g and 4 °C for 10 min. The supernatant was transferred and the protein concentration was determined by use of the Bradford technique (Bradford, 1976) with a commercially available reagent (Coumassie Protein Assay, Thermo Scientific, Rockford, IL). Lysates were adjusted to a protein concentration of 4 μg/μL and Western Blot analysis was performed with 6 % Tris–Glycine SDS–polyacrylamide gels, loading 40 μg of protein per well before transferring to a PVDF membrane and incubating with the primary antibody at 4 °C overnight. Antibodies were purchased from Cell Signaling Technology (Danvers, AKT, p-AKT [Ser473], mTor, and p-mTOR). All primary antibodies were diluted 5:1000 in TBST. Anti-rabbit HRP-linked secondary antibody was diluted 1:2000 (Cell Signaling

Technology). Blots were exposed to enhanced chemiluminescent substrate (Clarity Western ECL Substrate, Biorad, Hercules, CA), imaged sequentially (BioSpectrum Imaging System, UVP, Upland, CA) and densitometry was performed using VisionWorks software (VisionWorks LS software, v. 8.1.2, UVP, Upland, CA).

Measurement of liver TG

Hepatic TG measurement was previously described in detail (Fry et al., 2018). The TG of lipid extracts was measured via the Hantzsch condensation method and compared with a triolein standard curve (Folch et al., 1957). The TG was expressed as a percentage based on milligrams per milligram of wet weight.

Statistical analysis

Prior to all statistical analysis, five cows were removed from the study for the following reasons unassociated with the treatments and as follows; one animal (BCAA n=1) died as a consequence of an uterine prolapse; one animal (BCAA n=1) was removed from trial due to a respiratory disease; one animal (Control n=1) was unable to adapt to the tie stall; one animal (BCAAPG n=1) had an incorrect breeding date; and one animal (BCAA n =1) was anorexic for several days after calving and was unable to eat the treatment, thus confounding the data. The number of cows remaining for statistical analysis were: control (n=16), BCAA (n=14) and, BCAAPG (n=16).

DMI, energy balance (EB), protein balance (PB), milk yield, ECM, free AA, BHB, FFA, insulin, glucose, glucagon, liver enzymes, liver TG, and protein measurements were analyzed with repeated measurements ANOVA using PROC Mixed in SAS 9.4. Group, time and an interaction between group × time effects were included as fixed effects in all models. Postpartum outcomes on +5 and +21 days included the

actual sampling day time point -10 as a covariate. Block was included in every model as a random effect. Interaction terms were tested and were not included in the final model if the *P*-value was ≥ 0.05. Tukey's post hoc test was used for multiple comparison correction of *P*-values for all pairwise LMS comparisons.

Five covariance structures were tested for each outcome (simple, compound symmetry, autoregressive order 1, Toeplitz, and unstructured). Data from insulin, glucose, and free AA concentration in plasma were unequally spaced and therefore the covariance structures analyzed for each of the models were spatial power law, Gaussian and spherical. The covariance structure with the lowest Akaike's information criterion was selected. Normality and homoscedasticity of residuals was tested for each model fit. To meet the assumptions, the outcome variables BHB, FFA, free AA and glucose concentrations were log transformed. All results are presented as geometrical means and 95% CI.

Results

There was no difference in the parity among the cows enrolled in the three groups ($P = 0.82$). Average BCS at enrolment was 3.25 (3.0, 3.5) for the three groups. In the first week postpartum, BCS for control, BCAA and BCAAPG was 3.25 (3.0, 3.25), 2.75 (2.75, 3.25) and 3.0 (3.0, 3.25; $P = 0.44$). The BW lost (95%CI) from 1 wk before calving until 6 wk postpartum was 87.6 (65.8, 109.3), 77.9 (55.4, 100.5), and 71.7 (49.7, 93.6) for control, BCAA and BCAAPG, in that order ($P = 0.50$). Average BW at enrollment did not differ among the two treatments and control groups ($P = 0.36$) along with the BW during the first week postpartum ($P = 0.55$). The average days dry

was similar among all groups ($P = 0.37$).

Liver enzymes and hepatic TG

On average, the three biopsies were performed at days -9 ± 4.2, +5 ± 0.8, and 21 ± 1.2 for all cows in the three groups. Liver enzymes and hepatic TG % are presented in Table 5.1 and Figure 5.1. Cows in the BCAAPG group had less hepatic TG during the postpartum period compared with the control group. Postpartum, the control group had an increased concentration of AST and GLDH in plasma compared with BCAAPG group.

Table 5.1. Repeated measures ANOVA LSM (95% CI) for liver panel enzymes and hepatic triglycerides

		Treatments[1]			P-values		
		Control	BCAA	BCAAPG	Tx	Time	Tx × Time
Triglycerides %	-9 d	0.7 (0.4, 1.2)	0.5 (0.3, 0.9)	0.6 (0.3, 0.9)			
	+5 d	8.4 (5.3, 13.3)	7.0 (4.2, 11.5)	4.2 (2.5, 7.1)	0.02	0.0001	0.84
	+21 d	9.2 (5.8, 14.6)	7.5 (4.7, 11.9)	4.6 (3.0, 7.1)			
Total proteins g/dL	-9 d	7.1 (6.9, 7.3)	7.0 (6.8, 7.2)	7.1 (6.8, 7.2)			
	+5 d	6.5 (6.3, 6.7)	6.5 (6.3, 6.8)	6.6 (6.4, 6.8)	0.49	0.0001	0.33
	+21 d	7.2 (7.0, 7.4)	7.4 (7.2, 7.7)	7.3 (7.1, 7.5)			
Albumin g/dL	-9 d	3.6 (3.5, 3.7)	3.6 (3.5, 3.7)	3.6 (3.5, 3.7)			
	+5 d	3.5 (3.4, 3.6)	3.5 (3.4, 3.6)	3.5 (3.4, 3.7)	0.30	0.0003	0.95
	+21 d	3.6 (3.5, 3.8)	3.7 (3.5, 3.8)	3.7 (3.6, 3.9)			
Globulin g/dL	-9 d	3.5 (3.2, 3.7)	3.4 (3.1, 3.6)	3.4 (3.1, 3.6)			
	+5 d	3.0 (2.8, 3.2)	3.1 (2.8, 3.3)	3.1 (2.9, 3.3)	0.37	0.0001	0.33
	+21 d	3.6 (3.4, 3.8)	3.8 (3.5, 4.0)	3.5 (3.3, 3.8)			
Aspartate aminotransferase (AST) U/L	-9 d	71.9 (63, 82)	73.1 (64, 84)	68.8 (61, 78)			
	+5 d	112.2 (100, 125)	99.6 (88, 112)	94.2 (84, 105)	0.01	0.01	0.75
	+21 d	91.4 (82, 102)	84.8 (75, 96)	82.4 (74, 92)			
Sorbitol dehydrogenase (SDH) U/L	-9 d	4.2 (3.3, 5.4)	5.0 (3.8, 6.7)	4.7, (3.7, 6.0)			
	+5 d	4.7 (3.7, 5.8)	4.1 (3.2, 5.3)	3.6 (2.9, 4.6)	0.24	0.0001	0.66
	+21 d	6.3 (5.0, 7.8)	6.6 (5.1, 8.8)	5.7 (4.6, 7.1)			

Table 5.1. (Continued)

Glutamate dehydrogenase (GLDH) u/L	-9 d	25.4 (19, 34)	28.2 (21, 38)	25.4 (19, 34)			
	+5 d	30.5 (23, 41)	23.6 (17, 32)	20.9 (16, 28)	0.01	0.0002	0.97
	+21 d	48.9 (36, 66)	35.6 (26, 49)	32.3 (24, 43)			
Gamma-glutamyl transferase (GGT) U/L	-9 d	20.9 (17, 25)	24.1 (20, 28)	22.1 (18, 26)			
	+5 d	20.8 (18, 24)	19.4 (17, 22)	21.2 (19, 24)	0.18	0.0001	0.34
	+21 d	29.6 (26, 34)	24.7 (21, 29)	24.9 (22, 29)			
Alanine transaminase (ALT)	-9 d	34.8 (32, 38)	38.4 (35, 42)	35.6 (33, 39)			
	+5 d	27.9 (26, 30)	27.5 (25, 30)	26.9 (25, 29)	0.87	0.0001	0.52
	+21 d	30.7 (29, 33)	30.2 (28, 32)	31.4 (30, 33)			
Alkaline Phosphatase U/L	-9 d	32.5 (27, 38)	34.9 (29, 42)	36.0 (30, 42)			
	+5 d	32.5 (30, 35)	30.8 (33, 28)	29.4 (27, 32)	0.25	0.0001	0.61
	+21 d	27.0 (25, 29)	25.8 (23, 28)	26.1 (24, 29)			
Creatine Kinase U/L	-9 d	85.4 (68, 108)	85 (66, 109)	86.6 (69, 109)			
	+5 d	117.6 (94, 148)	117.2 (92, 150)	105.6 (84, 133)	0.79	0.52	0.37
	+21 d	123.5 (98, 155)	106 (83, 136)	133.5 (107, 168)			

[1] Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g/d of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCCAPG) received 550 g/d of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

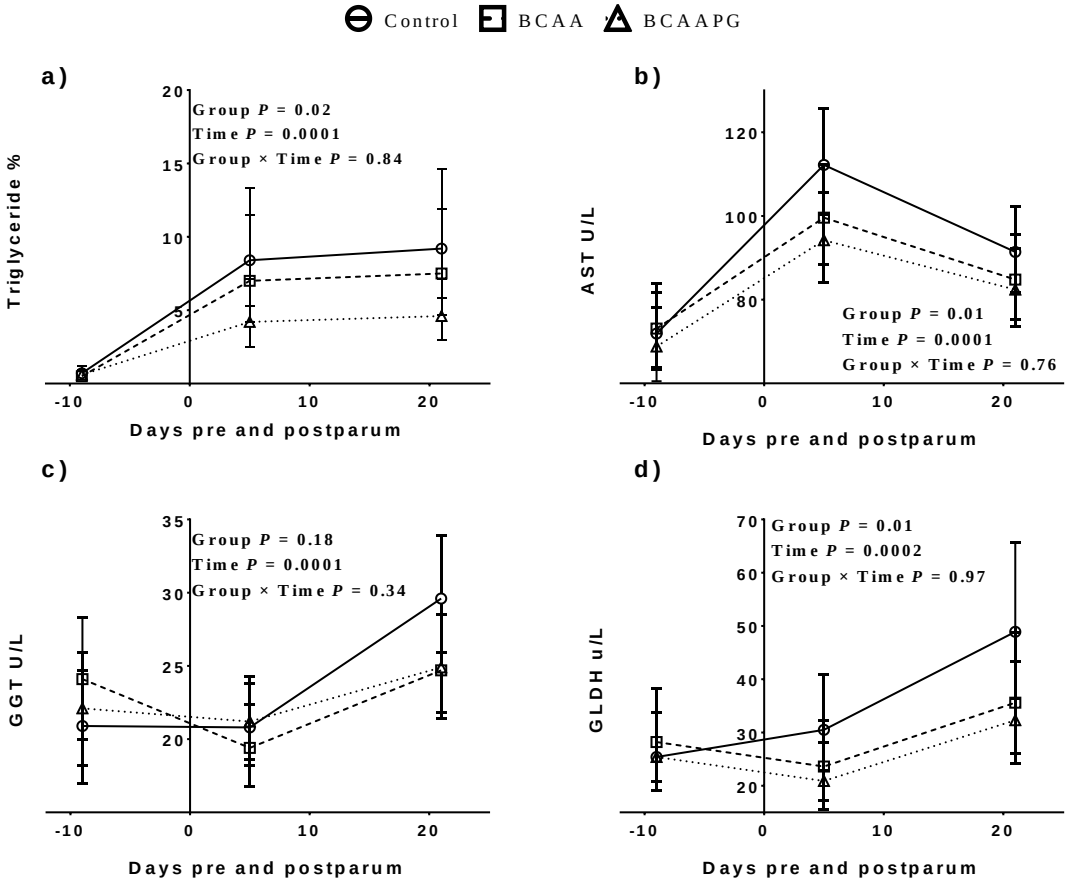

Figure 5.1. Least square means and 95% CI for ANOVA of a) hepatic triglyceride; b) GGT; c) AST; and d) GLDH. Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCCAPG) received 550 g per day of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM. Fixed effects were treatment group (Tx), time, and 2-way interactions; interactions with a P-value ≥ 0.05 were excluded from the model with the exception of Tx ×Time.

Production, EB and PB

Production level, energy and protein status of all animals included in this study are shown in Table 5.2. Cows in the BCAAPG group had a higher DMI than the control and BCAA groups during the first 35 DIM (P = 0.006). Cows in the control group had a lower milk yield and ECM than the two treatment groups. The EB and PB was similar among all groups during the first 35 DIM.

Table 5.2. Repeated measures ANOVA LSM (95% CI) for DMI, milk yield, ECM, energy balance (EB) and protein balance (PB)

Variable	Treatments[1] (95% CI)			P-values
	Control	BCAA	BCAAPG	Tx
DMI, kg	20.2 (19, 21)	20.9 (20, 22)	22.1 (21, 23)	0.006
Milk yield, kg	40.8 (39, 43)	43.1 (41, 45)	44.1 (42, 46)	0.05
ECM, kg	45.6 (43, 48)	48.6 (46, 51)	48.9 (46, 52)	0.05
EB %	75.9 (72, 80)	77.0 (73, 81)	80,0 (76, 84)	0.31
PB %	89.6 (86, 93)	91.7 (88, 95)	93.4 (90, 98)	0.24

[1]Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCCAPG) received 550 g per day of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

Blood metabolites and hormones

Glucose, insulin and glucagon concentration in plasma are presented in Figure 5.2. Postpartum, insulin plasma concentration was higher in the BCAAPG group compared with the control group (P = 0.002). Glucose and glucagon plasma concentrations were similar among the groups during the first 29 DIM. BHB, FFA, glucose, insulin and glucagon concentrations in plasma on the exact days of the biopsies are reported on Table 5.3. Free EAA (BCAA and Met) and non-essential AA (**NEAA**)

(Gln and Glu) concentrations in plasma are shown in Figure 5.3 and Figure 5.4. There was a significant increase in free Val, Met and Glu concentrations in plasma in both the BCAA and BCAAPG groups.

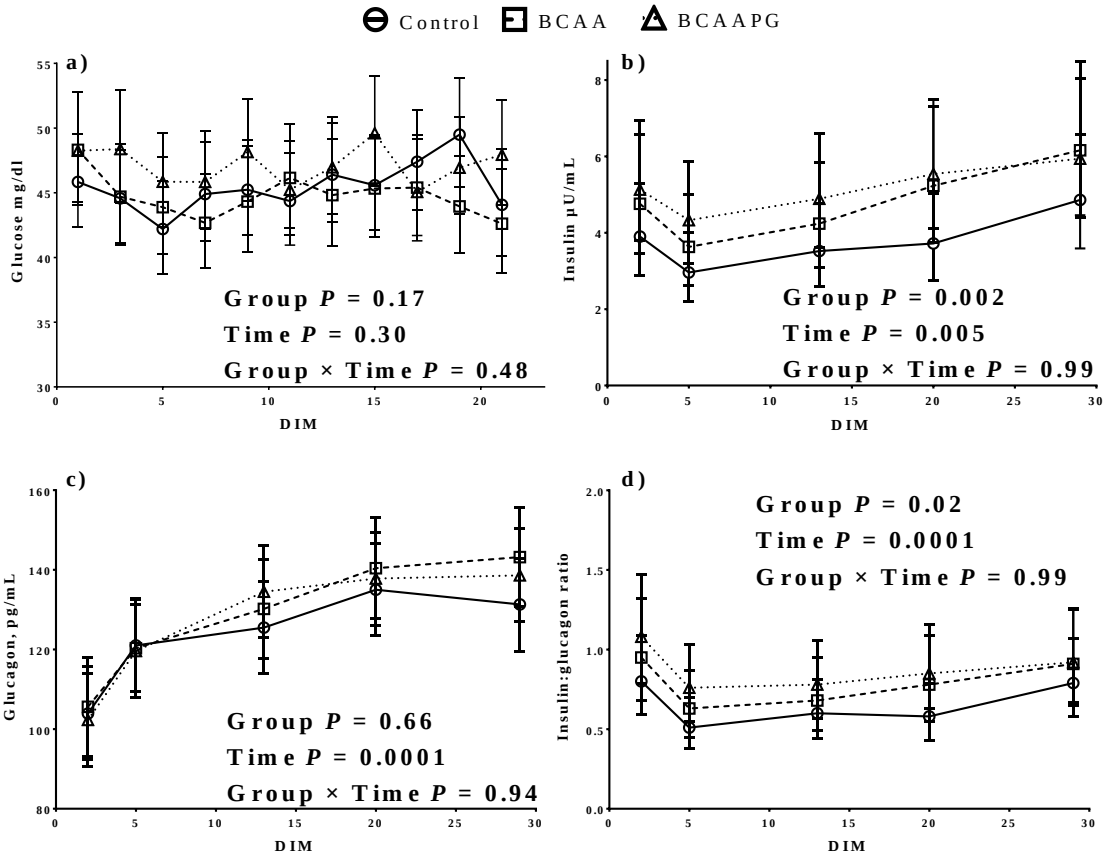

Figure 5.2. Least square means and 95% CI for ANOVA of a) glucose concentration, b) glucagon concentration, c) insulin concentration; and d) insulin:glucagon in plasma postpartum. Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCCAPG) received 550 g per day of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM. Fixed effects were treatment group (Tx), time, and 2-way interactions; interactions with a P-value ≥ 0.05 were excluded from the model with the exception of Tx ×Time.

Table 5.3. Repeated measures ANOVA LSM for β-hydroxybutyrate (BHB), free fatty acids (FFA) and glucose, insulin and glucagon on the day of the biopsies.

Variable	Treatments[1] (95% CI)			P-values
	Control	BCAA	BCAAPG	Tx
BHB 1-21 DIM (mmol/L)	1.1 (0.9, 1.1)	0.9 (0.8, 1.1)	0.9 (0.8, 1.1)	0.38
d -9	0.6 (0.5, 0.6)	0.6 (0.5, 0.6)	0.5 (0.5, 0.6)	0.79
d +5	1.2 (0.9, 1.6)	0.9 (0.7, 1.2)	0.8 (0.6, 1.0)	0.10
d +21	1.3 (0.9, 1.9)	1.3 (0.9, 1.9)	0.9 (0.7, 1.2)	0.16
FFA 1-21 DIM (µeq/L)	584.4 (492.6, 693.4)	555.8 (463.1, 666.8)	504.6 (425.2, 598.7)	0.42
d -9	199.5 (166.6, 238.8)	130.0 (109.8, 161.0)	160.9 (134.4, 192.8)	0.0032
d +5	769.9 (631.0, 928.7)	696.6 (529.1, 864.1)	520.1 (364.7, 675.6)	0.06
d 21	537.0 (407.6, 666.4)	515.3 (385.9, 644.7)	460.3 (346.8, 573.8)	0.63
Glucose 1-21 DIM (mg/mL)	45.4 (43.4, 47.5)	44.7 (42.6, 46.9)	47.1 (45.0, 49.3)	0.17
d -9	55.8 (52.8, 58.9)	55.2 (51.8, 58.5)	57.7 (54.6, 60.7)	0.33
d +5	48.6 (33.4, 63.8)	50.1 (35.4, 64.7)	52.1 (37.8, 66.3)	0.44
d +21	44.3 (40.1, 48.4)	44.6 (40.2, 48.7)	48.6 (44.9, 52.3)	0.21
Insulin 1-29 DIM (µU/mL)	3.7 (3.2, 4.4)	4.73 (4.0, 5.6)	5.1 (4.4, 6.1)	0.002
d -9	13.8 (10.2, 17.5)	16.4 (12.3, 20.4)	16.5 (12.9, 20.1)	0.50
d +5	3.9 (2.9, 4.9)	3.9 (2.9, 5.0)	4.5 (3.5, 5.5)	0.59
d +21	4.6 (2.8, 6.3)	6.3 (4.3, 8.3)	6.9 (4.4, 7.9)	0.33
Glucagon 1-29 DIM (pg/mL)	123.3 (115.4, 131.2)	127.9 (119.5, 136.4)	126.6 (118.7, 134.5)	0.66
d -9	119.9 (107.3, 132.5)	129.8 (116.3, 143.3)	121.2 (108.6, 133.8)	0.31
d +5	121.0 (118.7, 123.3)	120.6 (118.2, 123.4)	119.0 (116.8, 121.4)	0.40
d +21	133.7 (123.7, 144.5)	142.8 (130.5, 156.6)	135.1 (125.0, 146.0)	0.65

Table 5.3. (Continued)

[1]Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCCAPG) received 550 g per day of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

[2]Fixed effects were treatment group (Tx), time, period (P) and 2-way interactions; interactions with a P-value ≥ 0.05 were excluded from the model with the exception of Tx ×Time.

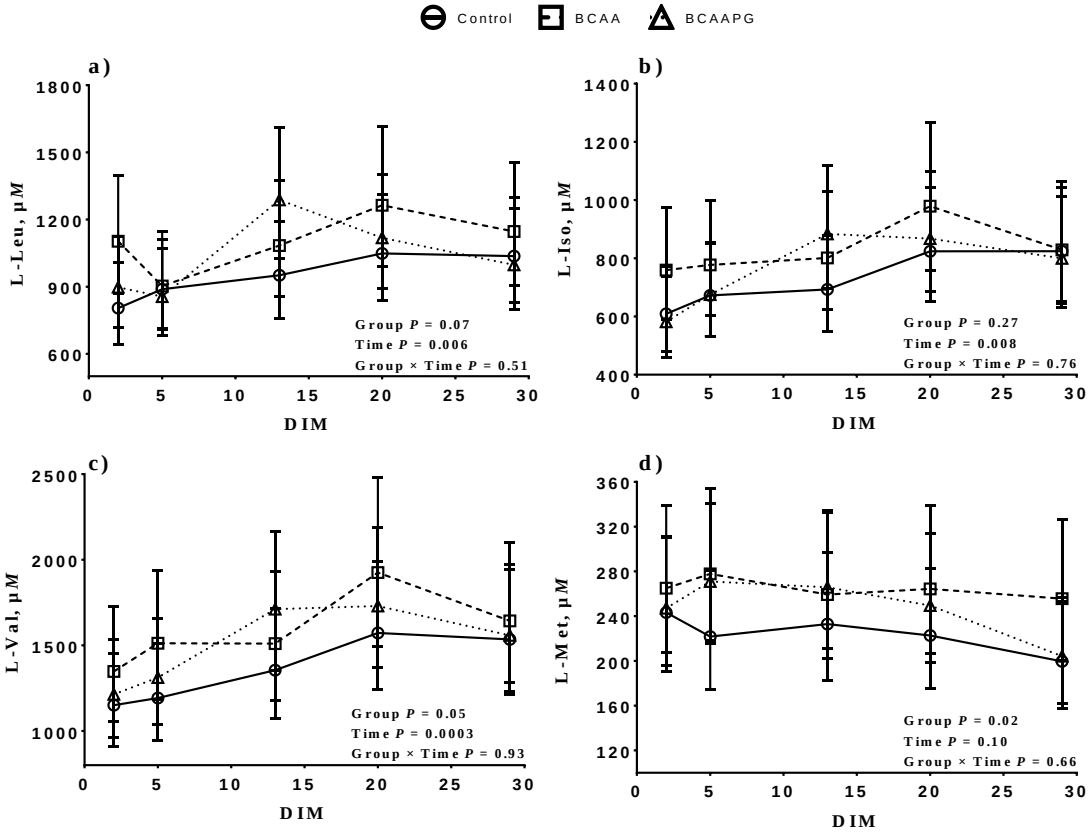

Figure 5.3. Least square means and 95% CI for ANOVA of a) free L-Leu; b) free L-Iso; c) free L-Val; and d) free Met concentrations in plasma postpartum. Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCCAPG) received 550 g per day of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM.

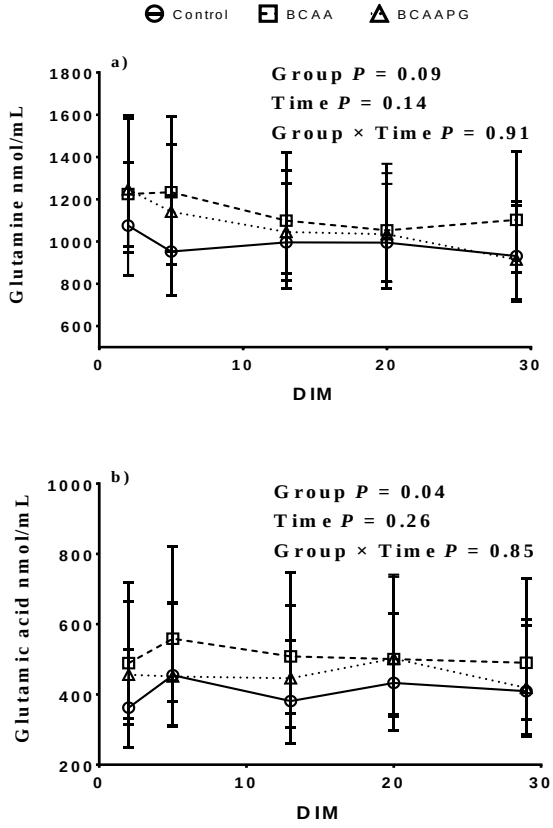

Figure 5.4. Least square means and 95% CI for ANOVA of a) free glutamine and b) free glutamic acid concentrations in plasma postpartum. Treatments: The control group received 200g of dry molasses from calving to 35 DIM, branched-chain amino acids (BCAA) received 550 g per day of rumen protected branched-chain amino acids mixed with 200g of dry molasses from calving to 35 DIM; and branched-chain amino acids plus propylene glycol (BCCAPG) received 550 g per day of BCAA mixed with 200g of dry molasses from calving to 35 DIM plus 300 ml of PG as oral drench from calving until 7 DIM. Fixed effects were treatment group (Tx), time, and 2-way interactions; interactions with a P-value ≥ 0.05 were excluded from the model with the exception of Tx ×Time.

Discussion

The objective of our study was to evaluate the effect of BCAA with or without PG oral administration on liver metabolism during the early postpartum period in Holstein dairy cows. Cows in the BCAAPG group had a lower concentration of TG in hepatic tissue during early postpartum. Hepatic lipidosis can be categorized based on the percentage of hepatic TG and is associated with acute BW loss, impaired immune response and reduced DMI, milk production and reproductive performance (Wensing et al., 1997, Hippen et al., 1999, Jorritsma et al., 2000).

Accumulation of TG in hepatic tissue is secondary to homeorhetic responses during early lactation in dairy cows. The DMI is reduced during late pregnancy and early postpartum due to physical and hormonal factors (McNamara et al., 2003, Janovick et al., 2011, Mann et al., 2015). Body fat reserves are mobilized as FFA, occasionally beyond hepatocyte use and clearance capacity. The degree of the TG infiltration in the hepatic tissue differs among individuals and is associated with the severity of the energy deficit (Geelen and Wensing, 2006). The excess storage of TG in the hepatocyte impairs the liver's metabolic function via cell lipotoxicity and mitochondrial dysfunction (Wang, 2014, Ringseis et al., 2015, Petta et al., 2016).

The concentrations of liver enzymes in plasma are a good indicator of liver or muscle injury, depending on the enzyme examined, and are commonly increased during early lactation in dairy cows (Imhasly et al., 2014). In our study, the BCAAPG group had a significant reduction in the concentration of AST and GLDH compared with the control group. The BCAA supplementation alone produced a moderate reduction in AST, GGT, and GLDH compared with the control group. The AST concentration is not

specific to the liver but has been correlated previously with liver damage in several species (Reid et al., 1983, Ekstedt et al., 2006) particularly when observed without alteration of CK which is a muscle specific enzyme. Previous studies have shown that supplementation with BCAA alone reduced hepatic TG in rodents and humans with nonalcoholic fatty liver (Muto et al., 2006, Arakawa et al., 2011, Honda et al., 2017) via suppression of fatty-acid synthesis expression and protein (Holeček, 2017) or upregulating the hepatic sterol regulatory element-binding protein/ liver x receptor pathway (Nishimura et al., 2010).

Oral administration of PG during 3 days postpartum did not produce a significant reduction in hepatic TG accumulation (Pickett et al., 2003, Mann et al., 2018). Cows in the BCAAPG group received PG oral drench from day 1 to day 7 postpartum and sustained a lower accumulation of hepatic TG through the first 21 DIM. Oral PG administration 10 days prepartum produced a reduction in total liver TG content during the first 21 DIM (Studer et al., 1993). This suggests that a period longer than 3 days of oral PG administration is needed to produce a substantial drop in TG accumulation in liver tissue during early lactation or there may be a synergistic action between the BCAA and PG to cause altered fatty acid metabolism and deposition.

However, PG oral administration for 3 days postpartum effectively reduced BHB and FFA concentration in dairy cows (Pickett et al., 2003, McArt et al., 2011, Gordon et al., 2017). Although our study did not find differences in BHB and FFA concentration after PG from day 1 until day 7 postpartum, BCAA supplementation in combination with PG increased insulin concentration in plasma during the first 29 days postpartum. This increment in insulin after PG oral administration is in agreement with

previous reports (Studer et al., 1993, Christensen et al., 1997, Miyoshi et al., 2001). Moreover, some AA can stimulate insulin secretion from pancreatic cells and regulate the action of insulin in peripheral tissues (Floyd et al., 1966, Henry, 1994, Menge et al., 2010). The gluconeogenesis from AA begins immediately after AA perfuses the liver tissue. The resulting hyperinsulinemia after AA infusion is a mechanism to regulate hyperglycemia induced by the glucogenic use of AA in the liver (Van Loon et al., 2003, Gadhia et al., 2013).

Diets rich in BCAA have shown a positive effect on other EAA and NEAA within the organism. The BCAA supplementation not only supplies substrates for glucose, Ala and Gln synthesis, but also acts as a regulatory factor for protein turnover (Ananieva et al., 2017, Holecek, 2018). Glutamine, Ala, Asp, and Pro might reduce ketogenesis and increase lipogenesis and glycogen synthesis in liver by activation of glycogen synthase and acetyl CoA carboxylase (Bai et al., 2015). In general, AA induce an anabolic response in the hepatocyte similar to the effect of insulin in peripheral tissues (Krause et al., 2002, Bifari and Nisoli, 2016). The reduction of hepatic TG and liver enzymes could be a synergic effect between BCAA supplementation, PG and increased insulin concentration in plasma in the BCAAPG group.

Glutamine and Ala are the major non-toxic ammonia transporters in dairy cows. The glutamine synthetase is an important step to detoxify ammonia via transformation to glutamine (Hakvoort et al., 2017). Almost all tissues can synthesize Gln but during growth and catabolic stages the cell increases Gln demand exceeding its stock within the organism. The concentration of Gln in plasma is reduced after calving as well as most of the AA (Doepel et al., 2006). In our study, the Gln plasma concentration for

BCAA and BCAAPG was higher than the control. This surplus of Gln might be favorable for the mechanism to dispose ammonia, especially in the muscle (DeBerardinis and Cheng, 2009).

We observed an increase in Met plasma concentration after BCAA supplementation. Interaction between BCAA and Met have been reported previously in different species (Langer et al., 2000). Studies using rumen protected Met supplementation in dairy cows produced both increased (Berthiaume et al., 2006) and reduced BCAA plasma concentrations (Guinard and Rulquin, 1995, Blum et al., 1999). Langer et al, (2000) suggested that the effect depends on the excess of BCAA over Met or vice versa, proposing a competitive inhibition metabolism, driving a surplus of Met in blood.

Conclusion

Hepatic lipidosis is one of the most common metabolic complications during early lactation in high producing dairy cows. Our study elucidates some of the effects of BCAA supplementation alone or in combination with PG as oral drench on liver TG content, liver enzymes and blood metabolites. We conclude that BCAA supplementation with PG might be a practical option to reduce hepatic lipidosis in dairy cows during early lactation. Moreover, BCAA supplementation in combination with PG influenced positively milk production and blood metabolites postpartum.

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CHAPTER 6

CONCLUSIONS

Worldwide, milk is one of the most produced and important agricultural products. The dairy industry directly provides income to roughly 150 million families also contributes to food security and access to protein for millions of people. Dairy production has progressed at an outstanding rate during the last decades due to innovation and research in different areas such as genetics, nutrition, reproduction, and preventive medicine. This growth in efficiency has permitted milk production to increase gradually, while reducing number of farms, dairy cows, and use of resources.

Dairy cattle face important physiological changes from late pregnancy to early lactation, including increased nutrient requirements due to milk production contemporaneous with diminished feed intake. The resulting period of negative energy balance (NEB) is a costly issue in dairy cattle leading to a high incidence of metabolic diseases during early lactation.

The objective of the study in Chapter 1 was to identify genomic regions associated with different concentrations of FFA, BHB and the development of HYK in longitudinally sampled Holstein dairy cow. Dairy cows have differing success in supporting their physiological functions while in energy deficit during the early postpartum period. Our results, showed the importance of serial measures of BHB and FFA for the genomic analysis of hyperketonemia as supported by another recent study showing increased genomic prediction accuracy of HYK when using serial measures. The 5 candidate genes of HSD17B10, HTR2C, ABCA1, ABCA2, and LIPC were identified based on genome-wide association to either HYK events or serial BHB

plasma/serum concentrations. Identification of genomic regions associated with different concentrations of FFA and BHB in early postpartum Holstein cows provides insight to an animal's genetic susceptibility to these conditions. This might allow us to reinforce preventative measures that decrease the incidence of hyperketonemia and improves genetic selection criteria.

The use of point-of-care (POC) for BHB concentrations in whole blood has become common tool for researchers and producers in the dairy industry. Laboratory methods for the determination of BHB are technically demanding and costly compared with the use of POC devices. The objective was to compare the accuracy and precision of the TaiDoc and Precision Xtra POC devices to measure BHB concentrations in bovine plasma samples at room temperature or 37°C with the laboratory gold standard method. Plasma BHB concentrations as measured by both meters were well correlated with the gold standard. The meters demonstrated good sensitivity and specificity compared with the gold standard method for classifying cows with hyperketonemia (BHB ≥ 1.2 mmol/L) when samples were measured at 37°C. Other studies aslo showed when using the POC devices as cow side test, the ambient temperature sample should be considered.

Increased consumption of branched-chain AA have shown advantageous results during catabolic stages in different species. The objective was to evaluate the effect of rumen protected branched-chain amino acids (BCAA; 375 g of 27% L-Leucine, 85 g of 48 % L-Isoleucine and 91 g of 67% L-Valine) with or without propylene glycol (PG) oral administration on milk yield, energy corrected milk (ECM), milk composition,

energy balance (EB), protein balance (PB), energy related plasma metabolites, liver enzymes and liver triglycerides (TG) during the early postpartum period in dairy cows.

Supplementation of branched-chain AA did not affect milk yield of dairy cows. However, BCAA alone decreased the number of plasma samples classified as hyperketonemic and increased concentration insulin and free valine in plasma as well as plasma and milk urea N. Supplementation of BCAA in combination with oral administration of PG reduce some liver enzymes, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), glutamate dehydrogenase (GDHL) and the percentage of hepatic triglycerides.

Our results, suggest that BCAA supplementation might alter the FFA metabolism. Also, the combination of BCAA and PG likely provides more substrates for gluconeogenesis, stimulating insulin secretion and reducing mobilization of FFA, and may enhance FFA utilization within the hepatocyte. Therefore, the use of RP-BCAA in combination with PG might be a feasible option to reduce hepatic lipidosis in dairy cows during early lactation.

Our study was deficient in a treatment group with only PG supplementation and therefore is unable to estimate the effect caused by PG alone. Regardless of this, a small number of dissimilarities between the BCAA and BCAAPG groups were observed, suggesting that PG alone was not responsible for detected changes in comparison with the control group. The dose of BCAA used during the present study was formulated based on previous studies using post-ruminal infusion of BCAA and casein in mid and early lactation in dairy cows. The amounts required to generate a significant effect on

production using BCAA supplementation during early lactation require more evaluation.

The supplementation with BCAA in other species has shown to improve muscle metabolism during catabolic stages such as aging. Skeletal muscle is the biggest AA reserve in the body therefore cows brake down muscle tissue to meet the metabolic protein and amino acids requirements during early lactation. Therefore, future research is needed to understand the effect of BCAA supplementation alone and in combination with PG administration.

A reoccurring observation from this study is that cows supplemented with BCAA alone had a reduce incidence of HYK during the early postpartum. Non-targeted metabolomics using liquid chromatography-mass spectrometry (LC-MS) helps to quantify changes in concentration of metabolites in response to internal and external stimulants Therefore, non-targeted metabolic profiling is an excellent tool to further investigate the complex cellular adaptation between late lactation and early pregnancy in dairy cows, including development of HYK.