BRANCHED CHAIN AMINO ACIDS OR BRANCHED CHAIN KETO ACIDS IMPROVED HEPATIC METABOLISM AND PRODUCTION IN EARLY LACTATION DAIRY COWS

By

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ABSTRACT

BRANCHED CHAIN AMINO ACIDS OR BRANCHED CHAIN KETO ACIDS IMPROVED HEPATIC METABOLISM AND PRODUCTION IN EARLY LACTATION DAIRY COWS

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Early lactation dairy cows undergo metabolic adaptations at the time of parturition and onset of lactation, increasing their risk of hepatic lipidosis. Effects of branched chain amino acids and branched chain keto-acids in early lactation were determined in 2 experiments. In experiment 1, primary bovine liver cells enriched with hepatocytes (PLEH) were exposed to media mimicking the circulating environment of early lactation cows without (FA) and with (BCKA) branched chin keto acid for 72h. The BCKA effectively decreased intracellular triglyceride accumulation in PLEH compared to FA. Expression of genes controlling lipid metabolism, such as decreased lipid uptake, increased β oxidation, increased antioxidant response, decreased apoptosis, and a recorded decrease in mitochondrial membrane potential was identified in the BCKA treatment. In the 2nd experiment, 36 multiparous dairy cows were enrolled on a randomized block design and were treated from 1 -21 days postpartum, with 1 of 3 abomasally infused treatments; 1) saline (CON); 2) BCAA, including 67 g valine, 50 g leucine, and 34 g isoleucine; and 3) BCKA, including 77 g ketovaline, 57 g ketoleucine, and 39 g ketoisoleucine. No alterations to body weight, body condition and dry matter intake were recorded between treatments. The BCAA infused cows produced increased milk, fat, and lactose yield relative to the CON. Abomasal infusion of BCKA decreased liver triglyceride compared to CON. In conclusion, branched chain keto-acids alter hepatic lipid metabolism and decreased hepatic triglyceride accumulation while branched chain amino acids alter milk production.

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KEY TO ABBREVIATIONS

AA: amino acid

BCAA: branched chain amino acid

BCAT: branched chain amino acid transferase

BCKA: branched chain keto acid

BCKDH: branched chain α keto acid dehydrogenase

BCS: body condition score

BHB: β-hydroxybutyrate

BW: body weight

DM: dry matter

DMI: dry matter intake

ECM: energy corrected milk

ETC: electron transport chain

FA: fatty acid

FCM: fat corrected milk

FITC: fluorescein isothiocyanate

FL: fatty liver disease

FSC: forward scatter

ICG: Internal control genes

Keto–isoleucine: 3-methyl-2-oxovaleric acid sodium salt

Keto-leucine: sodium 4-mehtyl-2-oxovalerate

Keto–valine: sodium 3-methyl-2-oxubutryrate

MP: metabolizable protein

mTOR: mammalian target of rapamycin

MUN: milk urea nitrogen

NAFLD: non-alcoholic fatty liver disease

NASH: non-alcoholic steatohepatitis

NEB: negative energy balance

NEFA: non esterified fatty acids

NEL: net energy of lactation

NPB: negative protein balance

NTC: non template control

PLEH: primary bovine liver cells enriched with hepatocytes

RDP: rumen digested protein

RUP: rumen undigested protein

SCC: somatic cell count

SSC: side scatter

TAG: triacyl glyceride

TMR: total mixed ration

TMRE: tetramethylrhodamine ethyl ester

VLDL: very- low density lipoproteins

$\Delta\Psi$: mitochondrial membrane potential

CHAPTER 1

REVIEW OF THE LITERATURE

LITERATURE REVIEW

Early Lactation Physiology

The dairy cow undergoes many physiological and metabolic changes at the onset of lactation and parturition. Nutrients that were partitioned to the gravid uterus during the prepartum period are redirected towards lactation and immune function in the immediate postpartum period (Bell, 1995; Drackley, 1999). To manage this nutrient partitioning, the dairy cow is under the control of both homeostatic and homeorhetic signaling. Homeostatic signaling is related to short term regulation of physiological functions while homeorhetic signaling is the adjustment of the body over longer periods of time to support a dominant physiological process, such as lactation (Bauman and Currie, 1980).

Negative Energy and Negative Protein Balance

The immediate postpartum period is of particular interest as it is the starting point for a productive and healthy lactation. This period is defined as the 3 weeks after parturition and is a period of metabolic and endocrine adaptation. With the onset of lactation, a dairy cow's nutrient requirements increase 30 – 50% compared to the last week of gestation (Bell, 1995), with a rapid increase in the demand for lipids, amino acids (**AA**), glucose, vitamins, and minerals. At the same time, a dairy cow decreases dry matter intake (**DMI**) about 30% the 24-48 h prior to calving and does not immediately recover DMI postpartum (Marquardt et al., 1976; Goff and Horst, 1997). This increased nutrient demand and inadequate DMI results in a negative energy balance (**NEB**) and negative protein balance (**NPB**). A prolonged period of NEB and NPB limits milk production and can lead to metabolic disorders as the cow deals with metabolic strain (Grummer, 1995; Drackley, 1999; Doepel et al., 2009; Gross et al., 2011).

During the first month of lactation, approximately a third of the energy required for milk synthesis is sourced from body reserves. To meet this need for energy reserves, adipose tissue will be lipolyzed into circulation in the form of non-esterified fatty acids (**NEFA,** Herdt, 2000). These NEFA will then be delivered to the mammary gland or to the liver for lipid synthesis and oxidation. (Bauman and Currie, 1980). Lipolysis and diminishing of body reserves can result in decreased measured body condition score (**BCS**, Bauman and Bruce Currie, 1980; Gross et al., 2011).

Hepatic Metabolism in Dairy cows

The liver acts as an essential organ in the maintenance of metabolic fuel, and is involved in the distribution of glucose, AA, volatile fatty acids, NEFA, and ketones, and must adjust to different metabolic adaptations in a NEB. Gluconeogenesis predominantly occurs in the liver from propionic acid and AA sources as a necessary function of ruminant metabolism. Glucose is an essential nutrient to the brain and mammary gland, but the ruminant animal absorbs very little glucose from their diet (Smith, 1971; Bell, 1995). The ruminant has little available digested carbohydrates and with decreased availability of plasma glucose in a NEB, the cow adopts glucose sparing metabolic strategies. Ketone bodies and NEFA are water soluble energy sources that can also be used for mammary FA synthesis, or as a fuel source in peripheral tissue. This use of ketones and NEFA is a metabolic strategy to compensate for insufficient intake of glucose precursors (Herdt, 2000; Drackley et al., 2001). However, as plasma NEFA increases, so does hepatic uptake of NEFA. When NEFA is metabolized in the liver, it can result in 1 of 3 outcomes: 1) fully oxidized in the citric acid cycle of the hepatocyte mitochondria to CO_2, 2) partially oxidized in the mitochondria to a ketone body, or 3) esterified and stored within the hepatocyte as a triacylglycerol (**TAG**; Drackley, 1999). Although hepatic TAG can be exported

out of the liver through very-low density lipoproteins (**VLDL**), the ruminant liver has a low capacity to secrete VLDL (Kleppe et al., 1988; Drackley et al., 2001). Consequently, when the influx of NEFA is higher than the demand or capacity for oxidation, TAG synthesis increases resulting in hepatic TAG accumulation.

Fatty Liver Disease

Hepatic lipidosis, also known as fatty liver (**FL**), is a metabolic disorder that effects the energy partitioning and health status of post-partum dairy cows. Fatty liver is diagnosed and categorized based on the severity of hepatic TAG accumulation. A normal liver is considered to have < 1% liver TAG as wet wight, whereas mild FL is 1 - 5%, moderate FL is 5 – 10%, and severe is FL > 10% TAG as wet weight. This evaluation and classification of FL is based on chemical or histological analysis. An estimate in 2004 determined that approximately 50% of cows would develop severe or moderate FL within the first month postpartum (Bobe et al., 2004). Fatty liver is associated with a higher risk for developing displaced abomasum, ketosis, and mastitis among other disorders (Drackley, 1999; Bobe et al., 2004). Lipid transport and secretion of lipoproteins decreases in animals with FL, affecting immune response and potentially leading to increased inflammation (Bobe et al., 2004). This physiological response drives a decrease in milk production, health status, and reproductive efficiency of dairy animals resulting in large losses for the dairy industry. Due to these adverse outcomes, it has been estimated that FL costs the U.S. dairy industry over $60 million per year in lost milk production, cow health, and overall profitability (Bobe et al., 2004).

To prevent FL, past studies have evaluated promoting the synthesis of VLDL to export hepatic TAG. These studies attempt to achieve this through choline and /or Met supplementation in the periparturient period and have yielded mixed results (Piepenbrink and Overton, 2003;

Cooke et al., 2007; Grummer, 2008). Due to the inconsistent results of these studies, and the ruminants innate inability to create sufficient levels of hepatic VLDL, (Kleppe et al., 1988; Drackley, 1999), other key factors should be targeted to prevent hepatic TAG accumulation. Strategies to mitigate FL should focus on: 1) decreased hepatic uptake of NEFA and 2) increased hepatic capacity for FA oxidation (Andersen et al., 2002). Our understanding of how these pathways interact with FL is limited in ruminants, as dairy scientists have focused on functional outcomes (Grummer, 1993) such as health and productivity (Bobe et al., 2004). Fatty liver disease has similar pathophysiological characteristics as a human disease called non-alcoholic fatty liver disease (**NAFLD**). This disease (also referred to as steatosis) is defined as excess retention of lipids within hepatocytes, mostly in the form of triglycerides, not due to alcohol use. Because there is significant experimentation of NAFLD in murine and human models, and the etiology of FL is similar, we evaluated and compared how development of FL may occur in dairy cows through similarities with NAFLD.

Non-alcoholic Liver Disease

Occurrence of NAFLD is diagnosed when lipid accumulation > 5% wet weight in liver tissue. Due to dysregulated lipid metabolism, accumulation of TAG can lead to a lipotoxic environment, and NAFLD can progress to non-alcoholic steatohepatitis (**NASH**) (Angulo, 2002; Geng et al., 2021). This lipotoxicity is noted as having abnormal intracellular signaling, organelle dysfunction, and chronic inflammation (Neuschwander-Tetri, 2017). In human medicine, there is extensive research of underlying metabolic mechanisms that can result in the development of NAFLD. Insulin resistance is considered the most reproducible factor for the development of NAFLD in humans.

Insulin

Insulin allows for tissue uptake of glucose, but can also be considered as a liporegulatory hormone (Herdt, 2000). Insulin resistance is defined as impaired insulin-mediated uptake in tissue and is highly involved with the liver adipose axis in NAFLD (Perla et al., 2017). In NAFLD, insulin resistance leads to the accumulation of lipids in hepatocytes through lipolysis of peripheral tissues and hyperinsulinemia (Angulo, 2002; Rhee et al., 2011). In dairy cows, plasma insulin concentration is inherently lower than humans (De Koster and Opsomer, 2013). Adipose and muscle tissue are sensitive to alterations to plasma insulin while the mammary gland will take up glucose independent to insulin concentration. It is also thought that early lactation cows enter a state of hypoinsulinemia as a response to support lactation in a NEB. Dairy cows with low plasma insulin levels reduce glucose uptake of insulin responsive tissues (such as adipose and muscle) and allow for increased glucose uptake in the mammary (Bauman and Elliot, 1983; Butler et al., 2003). This hypoinsulinemia is characterized differently than the hyperinsulinemia status of human NAFLD patients.

Along with decreased plasma insulin, early lactation dairy cows display insulin resistance in peripheral tissues to further this metabolic adaptation (De Koster and Opsomer, 2013). This "glucose sparing" effect is important for ensuring milk production but can result in the expense of further inducing FL. Increasing plasma insulin can help prevent lipolysis of adipose tissue, but also decreases hepatic FA oxidation capacity which may be necessary to prevent TAG accumulation (Andersen et al., 2002).

Insulin also effects lipolysis of adipose tissue. Hormone sensitive lipase increases the hydrolysis of adipose TAG, increasing circulating NEFA. Increased hepatic uptake of NEFA leads to the development of NAFLD and FL(Geng et al., 2021). In insulin resistant states, insulin

cannot suppress the activity of hormone sensitive lipase, which increased lipolysis (Tamura and Shimomura, 2005). Excessive lipolysis of adipocytes can also further exacerbate insulin resistance through hormones and pro-inflammatory cytokines and further the development of FL and NALFD (Donnelly et al., 2005; Perla et al., 2017; Geng et al., 2021).

Fatty Acid Uptake

In ruminants and other species, hepatic uptake of FA is proportional to the plasma concentration of NEFA (Andersen et al., 2002; Tamura and Shimomura, 2005). Ruminants have very low capacity for lipogenesis of FA in hepatic tissue as they have very low activity of enzymes ATP citrate lyase and NADP malate dehydrogenase (Bell, 1981). It has been demonstrated by Donnelly et al., 2005 that most of the liver TAG are derived from serum NEFA pool. The circulating NEFA concentration is proportional to TAG synthesis in the ruminant and in the postpartum period, when NEFA is aggressively mobilized, hepatic uptake of FA increases (Grummer, 1993). Plasma membrane protein transporters assist in the influx of FA into cell types such as hepatocytes (Musso et al., 2009). Transporters of interest include a class of fatty acid transport proteins (FATP). Hepatocytes can express FATP5, which with increased expression, can increase intracellular FA uptake through increasing membrane protein concentrations and alterating RNA transcription (Musso et al., 2009). In NAFLD patients, FA uptake is increased through FATP5 (Geng et al., 2021). Once these FA are taken up into the hepatocyte, they can be oxidized (fully to acetyl Co-A or partially to ketone bodies), or can be esterified into TAG for storage or export via VLDL (Rolo et al., 2012).

Fatty Acid Oxidation

Fatty acids can be oxidized by mitochondrial, peroxisomal, or microsomal oxidation. Fatty acid oxidation is the result of progressively oxidizing FA to acyl-CoA and finally acetyl-

CoA. This pathway allows for the electrons available within the FA to transfer to other substrates that may then be used in the electron transport chain and for the creation of acetyl CoA for use in the TCA cycle or in ketogenesis. Most FA will begin oxidation in the mitochondria, where the FA enters the mitochondria via the carnitine shuttle and activation of CPT1A on the mitochondrial membrane. Once inside the mitochondria, a class of enzymes called the acyl-CoA dehydrogenase will begin to sequentially oxidize the acyl-CoA to smaller chain lengths. Fatty acid chain length determines what specific enzyme of acyl-CoA dehydrogenase the FA will be catalyzed by. The second step uses a matrix soluble enoyl-CoA hydratase encoded by *ECHS1* to convert the enoyl-CoA to 3-hydroxyacyl-CoA. The third step entails enzyme hydroxyacyl-CoA encoded by *HADH* converting hydroxyacyl-CoA to 3-ketoacyl-CoA. Finally, the fourth step uses the enzyme medium chain 3-ketoacyl-CoA thiolase encoded by *ACAA2* converting 3-ketoacyl-CoA to acyl CoA and acetyl-CoA. From this point, acyl-CoA enters the TCA cycle or ketone generation (Goodridge and Sul, 2000; Houten and Wanders, 2010).

There are contradictory results as to whether FA β – oxidation increases or decreases in a state of NAFLD (Di Ciaula et al., 2021). Necessary FA oxidation is required to prevent the intracellular accumulation of TAG, but excessive FA oxidation can lead to oxidative stress and a reduction in antioxidants defense (Malaguarnera et al., 2009). In a murine model, disruption of a acyl-CoA oxidase leads to NAFLD (Reddy and Rao, 2006). Although there are discrepancies within understanding β-oxidation to FL, it is known that when β-oxidation is insufficient in hepatic tissue, lipids will accumulate(Di Ciaula et al., 2021).

Ketone bodies are synthesized when TCA intermediates become starved, and acetyl-CoA cannot enter the TCA cycle to be oxidized (Di Ciaula et al., 2021). Ketone bodies such as β hydroxybutyrate (**BHB**) and acetoacetate are formed in the liver to be used as water soluble

energy sources for extra-hepatic tissues. Production of ketones bodies is proportional to the rate of FA oxidation (Goodridge and Sul, 2000). Using the hepatic oxidation theory, the oxidation of fuels in the liver control feeding behavior and intake. Increasing hepatic oxidation through lipolysis and mobilization of NEFA can induce hypophagia. Ketone production will become elevated as intermediates of the TCA cycle are consumed from elevated oxidation (Allen et al., 2009). Hyperketonemia (ketosis) is a metabolic disorder resulting in negative health consequences and decreasing intake and milk production (Veenhuizen et al., 1991). The relationship between elevated hepatic oxidation and FL can indicate why intake decreases in severe FL. Because of its relationship to FA oxidation, the pathogenesis of ketosis and FL is similar, indicating the association between these diseases and to intake (Goff and Horst, 1997; Bobe et al., 2004).

Electron transport chain

The electron transport chain (**ETC**) is a system of transmembrane proteins that result in oxidative phosphorylation within mitochondrial cristae. The layout of the 4 oxidative phosphorylation complexes including complex V (ATP synthase), allows for the generation of a proton gradient to create a membrane potential for ATP generation (Zhao et al., 2019).

Mitochondrial complex I contains NADH dehydrogenase, which transfers electrons from the mitochondrial matrix NADH to ubiquinone, allowing for proton translocation (Zhao et al., 2019). A key protein subunit within this protein includes NADH: ubiquinone oxidoreductase subunit A9 encoded by *NDUFA9,* and, interestingly, NAFLD mice expressed less *NDUFA9* than controls (Pirola et al., 2013; Zeng et al., 2021). Mitochondrial complex II, contains an enzyme succinate dehydrogenase or SDH. The SDH complex plays a role in both the TCA cycle converting succinate to fumarate and in the reduction of ubiquinone in the ETC. The SDH

complex is made up of 4 subunits encoded by *SDHA*, *SDHB*, *SDHC*, and *SDHD* (Rasheed and Tarjan, 2018). Differential expression of these subunits may indicate a change in activity between the TCA and ETC (Zhao et al., 2019). Complex III is referred to as CoQ-cytochrome c reductase, and allows for the proton transfer to the intermembrane space (Zhao et al., 2019). In a mouse model, a protein subunit of complex III encoded by *UQCRQ* is significantly downregulated in NAFLD animals (Zeng et al., 2021). Mitochondrial complex IV, referred to as cytochrome c oxidase allows for the transfer of electrons from cytochrome C to O_2, and again allows for the proton transfer to the intermembrane space. (Zhao et al., 2019). Complex V, called F_1F_0 ATP Synthase, is used for the generation of ATP. This complex allows for the protons to pass from the intermembrane space to the matrix. Transferring these protons back to the matrix transfers the stored energy created by the proton gradient to a subunit of Complex V, which is then able to phosphorylate ADP to ATP (Zhao et al., 2019).

Decreased function and expression of enzymes in the ETC can result in FL and NAFLD. There are notable results that mitochondrial respiratory chain complexes are decreased in advanced liver disease and NASH patients (Geng et al., 2021). These sites of oxidation are also responsible for reactive oxygen species (**ROS**) production that also affects the function of the cell. A rodent experiment has demonstrated that defective ETC can be a result of mitochondrial epigenetic modification resulting in NAFLD, due to ETC ROS production (Pirola et al., 2013). Alterations to the mitochondrial ETC and decreased expression of ETC can promote mitochondrial dysfunction and liver disease.

Mitochondrial membrane potential

The energy substrate used by many cellular processes is ATP, and most ATP is produced from oxidative phosphorylation within the mitochondria. Oxidative phosphorylation is based on

the active transfer of protons across the inner mitochondrial membrane resulting in a negative net energy charge referred to as the mitochondrial membrane potential (ΔΨ). This membrane potential is the power source that is harvested for ATP synthesis within the mitochondria (Crowley et al., 2016; Zorova et al., 2018). To measure ΔΨ, a permeate dye will move across the mitochondrial membrane as a result of the charged gradient and will become fluorescent among excitation. Less active mitochondria have a lower membrane potential (Crowley et al., 2016; Sukumar et al., 2016).

A class of proteins called uncoupling proteins or UCPs exists within the inner mitochondrial matrix to uncouple the ETC from ATP synthase. In the hepatocyte, UCP2 does this through dissipation of the proton electron chemical gradient across the inner mitochondrial membrane (Cheng et al., 2003; Zhao et al., 2019). Reducing ATP production may seem inefficient, but decreasing the ΔΨ can decrease the production of free radicals (Brand, 2000). Upregulation of *UCP2* alters the mitochondrial membrane gradient and allows for more ETC capacity in the presence of energy substrates such as FA (Chavin et al., 1999; Thompson and Kim, 2004). Upregulation of *UCP2* also impairs oxidative phosphorylation in the ETC in patients with NAFLD (Chavin et al., 1999). Increasing expression of *UCP2* can decrease ROS generation but will concede capacity for ATP production within the ETC (Rolo et al., 2012).

Oxidative stress and antioxidants

Mitochondria are a major source of cellular ROS. Reactive oxygen species are generated from the ETC and are defined as free radicals that have a single unpaired electron promoting electron transfer through reduction-oxidation reactions (Jones and DeLong, 2000; Sordillo and Aitken, 2009). The amount of ROS generated within a cell can be beneficial as they play a role as secondary messengers of kinase signaling cascades, but large amounts of ROS can lead to

DNA damage, insufficient ATP generation, and cell death (Kaminskyy and Zhivotovsky, 2014; Zhao et al., 2019). In the case for FL, excessive FA oxidation can result in the generation of ROS and other free radicals.

Oxidative stress is defined as the imbalance between oxidants and their neutralizing counterparts, antioxidants. Oxidative stress typically occurs in the postpartum period of dairy cows (Reynolds et al., 2003). Similarly, increasing ROS is a characteristic of NAFLD and can increase inflammatory responses and cell death (Spahis et al., 2017). Reactive oxygen species can trigger lipid peroxidation, attack macromolecules, or induce the formation of cytokines including TNF – α, and IL–8 causing caspase activation and hepatocyte cell death. (Angulo, 2002).

Excessive ROS generation must be neutralized by antioxidants to limit the oxidation of macromolecules necessary for normal cellular function. Antioxidants can be synthesized enzymes or can be derived from the diet. Enzymes that can directly reduce ROS include glutathione peroxidase and superoxide dismutase (Jones and DeLong, 2000; Sordillo and Aitken, 2009). Decreased levels of antioxidants also may play a role in the development of FL. In a murine model, supplementing mice with antioxidants or antioxidant analogs decreased the oxidative damage to mitochondrial DNA and increased the activity of ETC units (García-Ruiz et al., 2013).Through promoting the antioxidant neutralizing capacity, we can reduce oxidative stress and FL.

Apoptosis

If antioxidant systems fail to balance ROS and result in oxidative stress, an apoptitic cascade can be triggered (Circu and Aw, 2010). Apoptosis is programmed cell death characterized by nuclear fragmentation, chromatin condensation, and cellular shrinkage. It can

occur as a normal result of development and aging but can also occur as a cellular response to disease damage or noxious agents, such as ROS and free radicals. The primary apoptotic pathway occurs through the activation of executioner caspases such as CASP3 and CASP7 (Aizawa et al., 2020). Apoptosis and cell turnover is low in a normal liver, but can be higher in NAFLD and is marker of liver disease progression to NASH (Luedde et al., 2014). Apoptosis is a low inflammatory pathway in comparison to other cell death processes. However, signaling and activation of these caspases can occur through DNA damage, ER stress, and ROS imbalance. Elevated levels of apoptosis are associated with liver disease and decreased liver function (Wang, 2014), and, in extreme cases, larger immune responses and liver fibrosis (Kanda et al., 2018).

Protein metabolism in early lactation

Along with lipid mobilization, the early lactation animal will also increase mobilization of AA from body reserves to meet lactation requirements (Bell, 1995). Protein mobilization in the early lactation is variable but is most markedly affected by inadequate DMI and increasing milk production. Amino acids are mobilized from the muscle to the mammary gland and other tissues (such as the involuting uterus) when nutrient demands from DMI are not met (Andrew et al., 1994). When in a NEB, AA are also used for glucose synthesis (Bauman and Currie, 1980). Compared to mid-lactation, the periparturient period leads to large increases in demands for rumen undegradable protein (**RUP**) and metabolizable protein (**MP**); (Doepel et al., 2009; Osorio et al., 2013). A decrease in protein available post-ruminally can drastically affect the total MP available to the animal. In terms of MP, RUP accounts for approximately half of the total AA flow into the small intestine(NRC, 2001; Zanton, 2017).

Nitrogen Cycling

Rumen degradable protein (**RDP**) is also influential in determining the microbial AA available for MP and urea use within the animal. Microbial AA profiles are like the AA profiles that are found in milk (Zanton, 2017). Ammonia (NH_3) can also be used by rumen microbes for the synthesis of AA. This ammonia can either come from dietary urea sources or from within the animal via the urea cycle. Ammonia is absorbed through the rumen epithelium and enters the portal vein. Ammonia found in the portal vein can be sourced from the diet or from endogenous sources and is then directed to the liver to be converted to urea (Reynolds and Kristensen, 2008). Ammonia levels in the portal vein increase with increasing N intake. When N levels are insufficient to meet protein synthesis within the animal, the ruminant can recycle non-protein N, such as NH_3, in the protein regeneration cycle (Houpt, 1959). This cycle allows the ruminant to recycle urea from the gastrointestinal tract, where it is hydrolyzed to NH_3 to be used by rumen bacteria. Excess NH_3 from the rumen is absorbed through the gut wall and enters the portal vein and other blood pools. The body must then convert the toxic NH_3 to non-toxic urea via the urea cycle. The urea cycle is interrelated with the citric acid cycle though fumarate and α-ketoglutarate, which allows for the amination and creation of AA (Xu et al., 2020). This urea can be used in the gastrointestinal tract, can be excreted in urine, or secreted in milk. Most regulating factors for urea transfer back to the gut are unknown (Reynolds and Kristensen, 2008).

Amino Acids in protein metabolism

Beyond non-protein nitrogen sources, cows will receive necessary N and amine groups through AA. Amino acids are vital nutrients which are the basic units of peptides and protein. There are 10 AA that cannot be synthesized by the animal and are referred to as essential (**EAA**) and must be acquired through the diet or from rumen microbial protein. Amino acids are needed

to produce milk protein, but they are also essential for other biochemical processes including the conversion of N between tissues. Amino acids are used in gluconeogenesis, oxidation, ureagenesis, and transamination reactions (Zanton, 2017). Different protein sources have different AA profiles. In an incomplete AA profile, dairy cattle may become limited by AA availability resulting in a reduction in milk protein secretion. The classical theory of single limiting AA has been proven to be insufficient to understand the AA requirements of lactating dairy cattle. The theory most recently utilized by The National Academies of Sciences Engineering, Medicine 2021 model includes a quantitative assessment based on the sum of each EAA needed to fulfill each designated metabolic function. To determine AA supply in ruminants, one must know the basal supply of AA from the diet as well as the AA that are available postruminally from microbial protein. The challenge with this method is understanding the variability of efficiency of AA use based on the energy supply and physiological status of the dairy cow (NASEM, 2021).

Branched chain amino acids

A subset of AA of particular interest are branched chain amino acids (**BCAA**) which make up over 20% of the AA weight found in milk protein (NASEM, 2021). Branched chain amino acids are classified as such based on their aliphatic side-chain carbon skeleton and are composed of the AA leucine, isoleucine, and valine. These BCAA are classified as essential as they are found in the mammal via dietary absorption and cannot by synthesized at the level needed for metabolism by the mammal's anabolic pathways. Amino acids are categorized as ketogenic or glucogenic based on the breakdown of their carbon skeleton to different intermediates of acetyl-CoA or the TCA cycle (Stipanuk and Watford, 2000). Leucine is ketogenic, forming acetyl co-A and acetoacetate, whereas valine is glucogenic forming succinyl

co-A, and isoleucine is both ketogenic and glucogenic (Nie et al., 2018). The oxidation of BCAA is self-regulating, based on equilibrium within the cell cytosol and the mitochondria (Harper et al., 1984).

Unlike other AA, BCAA are not catabolized in the liver, with most BCAAs instead entering circulation to reach peripheral tissue to be catabolized (Nie et al., 2018). In both the cytosol and mitochondria, BCAA can be catabolized with the reversible transamination process resulting in the production of respective α ketoacids catalyzed through branched chain amino acid transferase (**BCAT**). This transamination process allows the amino group (NH_3) to form glutamate and/or alanine to be transported for use elsewhere and used throughout the body as a "nitrogen shuttle" and for the synthesis of nitrogen compounds such as ammonia. The resulting product from BCAA de-amination is branched chain keto-acids (**BCKA**s). These BCKAs can then enter the bloodstream and be transaminated back into BCAA or continue to be catabolized in the liver (Harper et al., 1984; Holeček, 2020). After transamination, the second and irreversible step of BCAA catabolism is the oxidative decarboxylation of BCKA catalyzed by branched chain α ketoacid dehydrogenase (**BCKDH**). Subunits of BCKDH are found in the highest quantity in the liver in dairy cows, within the inner mitochondrial membrane, potentially representing the role that the liver has in regulating these catabolic enzymes. The homeostatic levels of BCAA are maintained by these first 2 catabolic steps by BCAT and BCKDH (Harper et al., 1984; Webb et al., 2019). After these two catalyzed steps, the different BCAAs/BCKAs are going to enter the ATP generation process through respective ketogenic or glucogenic pathways (Bonvini et al., 2018).

BCAA in FL

Branched chain amino acids have long been associated with immunomodulation. This class of AA plays an important role in energy and nutrient metabolism, but they also serve as signaling molecules that can regulate pathways associated with glucose, lipid, and protein synthesis among others (Nie et al., 2018). Circulating levels of BCAA are related to the synthesis of hepatic proteins that mark liver function (e.g. acute phase proteins). As a part of this, BCAA supplementation has been used to improve the nutritional status and health of some liver cirrhosis patients. In a study designed to induce liver cirrhosis in rats, oral administration of BCAA increased the survival rate of cirrhosis induced rats (Eguchi, 2021). The relationship between BCAA and glucose tolerance and insulin are complex, and there are some discrepancies between the role of BCAA and insulin and glucose regulation. Supplementing BCAA at specific levels can promote metabolic markers associated with insulin resistance, but at high blood BCAA levels, there may be negative effects of insulin resistance in monogastrics (Nie et al., 2018). In rats fed a high fat diet, leucine supplementation led to higher glucose tolerance and insulin sensitivity throughout tissue types (Liu et al., 2017a).

The mammalian target of rapamycin (**mTOR**) pathway is a central regulator for mammalian cell growth or hypertrophy, as well as an AA balance sensor (Zhenyukh et al., 2017; Nie et al., 2018). The BCAA Val increases phosphorylation of mTOR and increases milk protein when other AA are kept at a specific ratio (Dong et al., 2018). Leucine also stimulates protein synthesis through mTOR (Tischler et al., 1982; Nie et al., 2018). In mid-lactation dairy cows, supplementation of Ile and Leu significantly increased milk production and mammary plasma flow, likley through the activation of mTOR (Tajiri and Shimizu, 2018; Yoder et al., 2020). In contrast, rumen protected supplementation of BCAA in early lactation cows did not significantly

increase milk yield when supplemented alone, despite increasing total MP (Leal Yepes et al., 2021). The influence of BCAA on signaling pathways seems to be based on the dosage and metabolic status of the animal, but BCAA can interact with an array of tissue types to increase protein synthesis and potentially affect FL.

Branched chain ketoacids

When evaluating hepatic metabolism, BCKA are of interest as they impact the liver of ruminants more directly than BCAA. If BCAA are not used for protein synthesis, they can be catabolized and form glucogenic (Ile and Val) or ketogenic (Val and Leu) precursors. After BCAA are transaminated by BCAT to BCKA, the non-nitrogenous BCKA may also result in immunomodulatory actions. The metabolite BCKA may more directly affect liver metabolism than BCAA, as the ruminant liver has very low activity of BCAT. Instead, BCAT and the transamination process occur in muscle and adipose tissues (Harper et al., 1984; Nie et al., 2018). Although BCKA are lacking N, BCKA still have the ability to modulate mitochondrial function and protein metabolism (Dong et al., 2016; Nie et al., 2018). Feeding rumen soluble branched chain keto-leucine to lactating dairy cows resulted in elevated milk fat yield (Vandehaar et al., 1988). Meanwhile, feeding rumen protected branched chain keto-leucine has demonstrated increased average daily gain and fat deposition on ovine carcasses (Flakoll et al., 1991). It is unknown how branched chain keto-isoleucine and keto-valine impact ruminant metabolism. Although little is known about the direct impacts of BCKA on FL or NAFLD, BCKA are a metabolite of BCAA catabolism and can impact energy and protein modulation in different species (Nie et al., 2018).

Conclusion

The early lactation dairy cow experiences a multitude of metabolic adaptations at the onset of lactation and parturition (Bauman and Currie, 1980). These metabolic adaptations place the cow at increased risk of developing FL, which can alter productivity and health status (Bobe et al., 2004). Fatty liver disease results from multiple factors including increase lipolysis, altered mitochondrial function, and alterations to insulin concentrations (Geng et al., 2021). The addition of protein, specifically BCAA, can alter the nitrogen status, nutrient signaling pathways, and have immunomodulatory effects of the animal resulting in possible protein and FL mitigation benefits (Nie et al., 2018). The catabolism of BCAA to BCKA is of interest in clinical settings and within the dairy industry for their effects on FL.

CHAPTER 2

BRANCHED CHAIN KETO-ACIDS ALTER LIPID METABOLISM AND TRIACYLGLYCEROL CONTENT IN PRIMARY LIVER CELLS ENRICHED WITH HEPATOCYTES

ABSTRACT

Plasma branched-chain amino acids (BCAAs) are negatively associated with liver triacylglycerol (TAG) level in dairy cows in early lactation. While a seminal study demonstrated that BCAAs have the lowest rate of removal by liver in dairy cows under various nutritional and physiological conditions, their transamination products, branched-chain α-keto acids (BCKAs), are mostly catabolized in the liver. Our objectives were to determine if BCKAs reduce intracellular TAG content and alter expression of genes related to lipid metabolism in primary liver cells enriched with hepatocytes (PLEH). PLEH were isolated from 3 nonpregnant mid-lactation multiparous Holstein cows previously. A customized medium (CM) was created to mimic the profile of circulating AAs, glucose, insulin, choline, and albumin levels observed for dairy cows on d 4 postpartum. Treatments were **FA** (CM + 1mM FA cocktail mimicking circulating FAs), and **BCKA** (FA + 33% of BCKA of corresponding circulating BCAA concentration). After 72 h, neutral lipids were stained with a fluorescent dye to quantify intracellular TAG content using flow cytometer. Expression of genes controlling lipid metabolism was also quantified in PLEH after 72 h. Treatment effects were determined using PROC MIXED in SAS. In the presence of FA, BCKA effectively reduced intracellular TAG level in PLEH (1111 vs 670, SEM = 73; $P < 0.01$). BCKA led to lower *FATP5* (hepatic long chain FA transporter; 0.459 vs 0.351, SEM = 0.034; $P = 0.07$) expression in PLEH. In response to BCKA, expression of β-oxidation gene (*ECHS1*) was also greater compared with FA (0.177 vs 0.231, SEM = 0.013; $P = 0.03$). Additionally, BCKA also led to higher expression of *UCP2* (0.345 vs 0.442, SEM = 0.022, $P = 0.02$) and lower mitochondria membrane potential (2274 vs 1900, SEM = 289, $P < 0.01$). Genes controlling antioxidant response (*GSR*, 16.2 vs 6.56, SEM = 2.18, $P = 0.02$; *KEAP1*, 0.943 vs 0.712, SEM = 0.043, $P = 0.01$) and apoptosis (*CASP3*, 0.757 vs 0.545, SEM = 0.057 $P = 0.04$; *CASP7*, 0.374 vs 0.208, SEM = 0.054,

P = 0.07) were decreased in response to BCKA, suggesting enhanced antioxidant defense compared with FA. We conclude that BCKAs altered lipid metabolism and reduced intracellular TAG level in PLEH.

INTRODUCTION

Fatty liver disease affects approximately 50% of all dairy cows in the early lactation, costing the U.S. dairy industry over $60 million annually in lost milk production, cow health, and reduced profitability (Bobe et al., 2004). Fatty liver disease (**FL**) is defined as the excessive accumulation of lipids in hepatocytes. Accumulation of triglycerides (**TAG**) in liver can lead to a lipotoxic state resulting in compromised liver function and subsequent negative impacts on health and productivity in dairy cows (Grummer, 1993; Bobe et al., 2004; Geng et al., 2021). Typically, FL occurs when dietary intake cannot meet energy demands at the onset of lactation and results in a negative energy balance (**NEB**) (Goff and Horst, 1997; Herdt, 2000). This NEB results in the lipolysis of adipose tissue and the mobilization of non-esterified fatty acids (**NEFA**) (Drackley, 1999). Although NEFA taken up by the liver has the potential to be oxidized or secreted, NEFA overload around parturition usually results in increased liver TAG accumulation in early lactation (Emery et al., 1992).

Current nutrition strategies aim at alleviating FL through enhancing TAG export through increasing very-low density lipoproteins (**VLDL**) synthesis by way of choline supplementation (Grummer, 1993). However, this strategy is not consistently effective as ruminants have a low capacity to secrete VLDL (Kleppe et al., 1988; Piepenbrink and Overton, 2003; Zhou et al., 2016b). Alternative strategies to reduce hepatic accumulation of TAG could include increasing hepatic lipolysis, lipid oxidation and decreasing fatty acid uptake. However, limited nutritional

preventative measures are developed to reduce the risk of fatty liver in dairy cows during early lactation utilizing these strategies.

Studies in humans and rodents demonstrated that branched-chain amino acid (**BCAA**) administration decreased liver TAG content and improved liver function (Arakawa et al., 2011). Administration of BCAA also significantly improved quality of life in human patients with advanced liver diseases (Tajiri and Shimizu, 2018). Such benefits observed in human and rodents likely originates from alterations in hepatic lipid metabolism, as BCAA administration was shown to regulate FA synthesis, transport, oxidation, lipolysis and adipokine secretion in the liver (Arakawa et al., 2011).

Plasma BCAA have an inverse relationship with FL; animals with elevated liver TAG had decreased plasma BCAA compared to animals with lower liver TAG. This negative association has been known for 20 years (Lobley, 1992), but the causal relationship between BCAA metabolism and fatty liver has not been explored. This may be due to a seminal study in the field demonstrating that BCAAs have the lowest rate of removal by liver in dairy cows under various nutritional and physiological conditions (Heitmann and Bergman, 1980). While there is no net uptake of BCAA in cow liver, over 75% of the transamination products of BCAAs – branched chain ketoacids (**BCKA**s) – are metabolized in the liver after initial transamination in peripheral tissues (e.g. muscle and adipose tissue) (Pell et al., 1986). Considering that there is no net uptake of BCAAs in the liver of dairy cows (Heitmann and Bergman, 1980), the BCAA relationship to FL may originate from BCKAs released from BCAA catabolism in extra-hepatic tissues. We therefore hypothesize that increased BCKAs decrease hepatic lipid accumulation by altering liver lipid metabolism, antioxidant response, and apoptosis in dairy cow hepatocytes exposed to fatty acids. To test this hypothesis, we established an in vitro model that closely mimics nutrients and

hormones that hepatocytes are exposed to in the first week of lactation using primary bovine hepatocytes isolated previously from adult cows (Zhou et al., 2018). We then used this model to determine the effects of BCKAs supplementation on intracellular TAG accumulation and expression of key genes regulating lipid metabolism, antioxidant response, and apoptosis.

MATERIALS AND METHODS

Bovine Primary Liver cells enriched with hepatocytes

Primary bovine liver cells enriched with hepatocytes (**PLEH**) were harvested via puncture biopsy from 3 non pregnant mid-lactation multiparous Holstein cows approximately 160 DIM free of clinical and subclinical disease (Zhou et al., 2016b). A nonperfusion technique (Lemley and Wilson, 2010) was used with 1-3 g of liver tissue harvested to isolate PLEH. PLEH from one of the three cows were used for this experiment in quadruplicate. Identify and functionality of PLEH were confirmed previously with expression of hepatocyte markers including albumin secretion, *BHMT* and *CYP1A1* (Zhou et al., 2018). The PLEH was thawed from stock stored in liquid N and resuspended in growth media. Isolated PLEH were seeded in 6-well plates with 4×10^5 cells per well. Three hours post seeding cells were switched to a maintenance medium [DMEM, catalog no. 31885–049, Thermo Fisher Scientific] supplemented with 0.2% fatty-acid-free BSA, 2 mM sodium pyruvate, 0.1 μM dexamethasone, 1 nM insulin, and 1% penicillin/streptomycin until 70% confluence was achieved. To closely mimic nutrients and hormones hepatocyte are exposed to *in vivo* in early lactation, we customized 20 AAs, glucose, choline, insulin, and albumin concentrations in DMEM to circulating levels (coccygeal vein) observed in Control cows 4 d postpartum (Zhou et al., 2016b). The concentrations of customized components are listed in Table 1. Using average circulating FA concentrations in Control cows on d 4 postpartum from a previous study (Zhou et al., 2016a) , a 1 mM FA cocktail is also created to mimic circulating FA

concentrations in cows during the first week of lactation. The FA cocktail is comprised of 23.0% C16:0, 23.0% C18:0, 19.7% C18:1, 29.0% C18:2, and 5.3% C20:4.

Treatments

Preliminary analysis using data from a previous study showed that at 7 d postpartum, circulating BCAA concentrations in cows with high liver TAG (50.83 ± 2.15 mg/g mg/g) are on average 33% higher than cows with low TAG (7.84 ± 1.55) (Supplemental Figure S.1). Although there is no net uptake of BCAAs by liver in dairy cows (Heitmann and Bergman, 1980), BCAAs are converted to BCKAs in extra-hepatic tissues and most BCKAs are shuttled to the liver for oxidation *in vivo* (Pell et al., 1986). Therefore, BCKAs at 33% equivalent of corresponding plasma BCAAs were chosen to mimic physiological levels of BCKAs. Treatments for this study were: 1) customized medium + 1mM FA cocktail (**FA**) (Figure 2.1; Zhou et al., 2016a); and 2) FA + BCKAs at 33% equivalent of corresponding plasma BCAAs on d 4 postpartum (**FA + BCKA**). Branched chain keto-acid components were sodium 4-methyl-2-oxovalerate (**keto-leucine**), 3-methyl-2-oxovaleric acid sodium salt (**keto-isoleucine**) and sodium 3-methyl-2-oxubutryrate (**keto-valine**) (Sigma Aldrich). For all treatments, cells were harvested at 72 h of culture.

RNA extraction and RT-qPCR analysis

Total RNA was extracted with Zymo Quick-RNA Mini prep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The RNA concentrations and integrity were determined by the NanoDrop One spectrophotometer (NanoDrop Technologies, Wilmington, DE). Samples were then prepared for RT-qPCR and diluted to 100 ng/µL with RNase free water in preparation for cDNA synthesis. Reverse transcription was performed with High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) according to manufacturer's instructions. Samples were analyzed in triplicate and a 5-point relative standard

curve with the non-template control (**NTC**) with each sample to test the relative expression level. Within each well, 4 µL of diluted cDNA combined with 6 µL of mixture composed of 5 µL 1×SYBR Green master mix (Applied Biosystems), 0.4 µL each of 10 µM forward and reverse primers, and 0.2 µL of DNase/RNase-free water were added. The qPCR was conducted in QuantaStudioTM 6 Flex Real-time PCR system (Applied Biosystems, Waltham, MA) with these conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C (denaturation), and 1 min at 60°C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 s, then 65 °C for 15 s. The threshold cycle (Ct) data were analyzed and transformed using the standard curve with QuantaStudio 6 Flex Real-time PCR system (Applied Biosystems, Waltham, MA; (primers reported in Supplemental Table S.1). Data was normalized with the geometric mean of the three Internal Control Genes (**ICG**) beta actin, *GAPDH*, and *RPS9.*

Fluorescent Microscopy

Cells were observed through a fluorescent microscope. Cells plated in 12 – well plates were fixed at 72 h exposed to treatment. The culture media was removed, and the cells were rinsed with PBS to remove non-adherent cells. The cell nucleus was stained with 1 ng/mL Hoechst 33342 blue (Cat # 33342, ThermoFisher Scientific, United States) and intracellular TAG stained with 20 ng/mL BODIPY® 493/503 (ThermoFisher Cat# D3922) lipids fluorescent dye. The PLEH and fluorescent stain were incubated at 37°C for 15 minutes then rinsed 3 times with 0.1 M phosphate buffer solution (**PBS**, ThermoFisher) before imaging using Nikon Eclispe Ts2-FL inverted fluorescence microscope equipped with a Nikon DS-FI3 5.9 Megapixel Color Camera. Single channel images were captured and converted into overlay images.

Flow Cytometry

Seventy-two hours after receiving treatments, cells were trypsinized, washed once with PBS, and stained with 1 mL PBS containing 20 ng/mL BODIPY® 493/503 lipid fluorescent dye for 15 minutes at room temperature. Cells were washed 3 times with PBS and analyzed with a BD LSR II cytometer (BD Biosciences, United States) using forward scatter (**FSC**) and side scatter (**SSC**) signals to target and gate hepatocytes. BODIPY® was excited with a 488 nm laser, and green fluorescent emission signals were collected in fluorescein isothiocyanate (**FITC**) channel (530/30 nm). The FITC signal was used to quantify mean fluorescent intensity (**MFI**) of the intracellular TG. Flow analyses were collected with a low flow rate and 10,000 events per sample were collected. No compensation was performed.

Mitochondrial Membrane Potential

Mitochondria membrane potential is measured using a cell-permeable, cationic dye tetramethylrhodamine ethyl ester (**TMRE**). The TMRE accumulates in the mitochondria matrix based on mitochondrial membrane potential (Crowley et al., 2016). Cells were cultured in 96-well black wall plates with low background fluorescence (Corning, AZ) at density described above. Seventy-two hours after treatments, culture medium was removed, and the wells were rinsed with PBS to remove non-adherent cells. Cells were then incubated with 100 nM TMRE for 30 min. Cells were rapidly washed three times with PBS before measurements were taken in fluorescence intensity well-mode (excitation 530 nm and emission 580nm) on a fluorescence microplate reader (SpectraMax M5, Molecular Biosciences).

Statistical Analysis

All data were analyzed using the mixed model procedure of SAS v9.4 (SAS Institute, Cary, NC) with the model: $Y = \mu + t_i + w_i + e_i$ where Y = the dependent variable, μ = overall mean, t_i =

fixed effect of treatment, w_i = random effect of cell replicate and e_i= residual error. Normality was tested using Shapiro-Wilk test. When normality assumption was rejected, data was log transformed for statistical analysis and back transformed for interpretation. Tukey-Kramer studentized adjustments were used for multiple comparisons to separate treatment means. Data are reported as least squares means with associated standard errors. Significant effects are declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

RESULTS

Intracellular TAG Accumulation in PLEH

As expected, substantial TAG accumulated in the cytosol of PLEH when exposed to FA similar to that of an early lactation cow (Figure 2.1A, green). In contrast, minimal TAG accumulation was observed by 72 h when FA + BCKAs were supplemented (Figure 2.1B). To quantify intracellular TAG accumulation, each PLEH was scanned using flow cytometry. In agreement with fluorescent microscopy results, FA + BCKA supplementation decreased intracellular TAG accumulation in PLEH by 40% ($P < 0.01$) compared to the FA at 72 hours (Figure 2.1C-F).

Expression of Lipid Metabolism Genes

With significantly lower intracellular TAG accumulation, changes in lipid metabolism within PLEH are expected in response to FA + BCKA treatment (Table 2.2). The FA + BCKA did not change expression of fatty acid translocase cluster of differentiation (*CD36)*, fatty acid binding protein 4 *(FABP4*), or microsomal triglyceride transfer protein *(MTTP)*. However, fatty acid transport protein 5 (*FATP5*) expression tended to be lower ($P = 0.07$) in response to FA + BCKA compared with FA. Additionally, expression of enzymes regulating fatty acid oxidation were also altered, as *ECHS1,* catalyzing the second step of the mitochondrial fatty acid beta-oxidation

pathway, was increased by FA + BCKA treatment compared with FA. There was no change in expression acetyl-CoA acyltransferase 2 (*ACAA2),* acyl-CoA oxidase 1 *(ACOX1),* hydroxyacyl-CoA dehydrogenase *HADH,* and peroxisome proliferator activated receptor alpha (*PPARα)* between treatments ($P > 0.14$)

Antioxidant Response, Mitochondria Membrane Potential, and Apoptosis

Mitochondrial membrane potential (ΔΨ), genes regulating antioxidant response and apoptosis are reported in Table 3. The FA + BCKA treatment increased expression of uncoupling protein 2 (*UCP2*) compared with FA, suggesting increased uncoupling activity of the mitochondrial membrane, and allowing for the flux of protons back to the mitochondrial matrix. Consequently, ΔΨ was lower in FA + BCKA treated PLEH compared with FA. The FA + BCKA treatment also decreased the expression of genes regulating antioxidant response including glutathione-disulfide reductase (*GSR, P* = 0.02) and Kelch-like ECH associated protein 1 (*KEAP1, P* = 0.01) compared to FA. Expression of glutamate-cysteine ligase catalyst subunit (*GCLC)* and nuclear factor erythroid derived 2 like 2 *(NFE2L2)* did not differ between treatments ($P > 0.20$). With activated antioxidant response, genes associated with cell apoptosis caspase-3 (*CASP3, P* = 0.04) and caspase-7 (*CASP7, P* = 0.07) were also downregulated by FA + BCKA treatment compared with FA.

DISCUSSION

BCKA Decreased Intracellular TAG Accumulation in PLEH

Increased liver TAG content is typically associated with decreased liver function in dairy cows around parturition, and obese cows are especially susceptible to TAG accumulation and compromised liver function during early lactation as they have a greater decrease in intake and hence more adipose tissue lipolysis (Bobe et al., 2004). While liver TAG is an indirect

measurement of hepatocyte function, and individual cows respond differently to the same level of liver TAG content, intracellular TAG content remains the standard for evaluating the effectiveness of fatty liver treatment and prevention strategies (Bobe et al., 2004).

In this study, we observed that FA + BCKA decreased the content of intracellular TAG in hepatocytes 40%. We suggest that FA + BCKA may minimize the risk of TAG accumulation and FL in dairy cows during early lactation decreasing health and production loss associated with FL. Considering BCAAs are not taken up by the liver and that BCAAs can be effectively converted to BCKAs in extra-hepatic tissues (e.g., muscle and adipose tissue), BCAA supplementation may also be able to effectively reduce liver TAG accumulation in cows.

Effect of BCKA on Hepatic Lipid Metabolism

Previous work with BCKA has demonstrated that feeding keto-leucine increased weight gain and altered fat deposition on lamb carcasses (Flakoll et al., 1991) and increased milk fat yield in lactating cows (Vandehaar et al., 1988) suggesting that BCKA may impact lipid metabolism in extra-hepatic tissues. Although the impact of FA + BCKA supplementation on liver lipid metabolism has not been evaluated, it is expected that alterations in various aspects of lipid metabolism likely also contribute to the significant decrease in intracellular TAG level in response to FA + BCKA supplementation.

Long chain fatty acids can enter hepatocytes through passive diffusion. With the high circulating NEFA concentration in early lactation, hepatic fatty acid uptake is believed to be primarily regulated by circulating fatty acid concentrations. However, similar to extra-hepatic tissues, fatty acid transport proteins (FATP) are also expressed in hepatocytes and facilitate fatty acid uptake (Bradbury, 2006). Hence, the tendency towards decreased expression of *FATP5* in FA

+ BCKA treated PLEHs suggests a decrease in long-chain fatty acid uptake into hepatocytes, thereby contribute to the lower liver TAG accumulation.

The liver is a major organ that removes NEFA from circulation for oxidation, so the capacity of fatty acid oxidation in liver is crucial in preventing TAG accumulation in hepatocytes. Therefore, we expected that genes relating to enzyme function in fatty acid oxidation would be upregulated in response to FA + BCKA supplementation. However, we found that neither *ACOX1* nor *HADH* were upregulated. *ACOX1* catalyzes the rate-limiting step of peroxisomal beta-oxidation of straight-chain very-long-chain fatty acids and *HADH* catalyzes the third step of the beta-oxidation of medium and short-chain 3-hydroxy fatty acyl-CoAs. On possible reason these genes were not upregulated is that cells were harvested at 72 h after initial exposure to fatty acids, by which time *PPARA* target genes regulating beta-oxidation may have already successfully oxidized sufficient fatty acids to prevent intracellular TAG accumulation. Similarly, although *PPARA*, the master regulator for fatty acid oxidation, did not respond to FA + BCKA treatment, the significant increase in *ECHS1* expression suggests that FA + BCKA may reduce hepatic TAG accumulation by enhancing β-oxidation (Houten and Wanders, 2010; Sharpe and McKenzie, 2018). Evaluation of enzyme concentration and rate of oxidation within these treatments would further support the treatment effects on FA oxidation.

Antioxidant Response, Mitochondria Membrane Potential, and Apoptosis

Oxidative stress is defined as the imbalance between oxidants and antioxidants. In transition dairy cows, metabolic stressors and onset of disease may increase oxidative stress due to elevated oxidation of energy substrates (Goff and Horst, 1997; Sordillo and Aitken, 2009). Consequently, production of reactive oxygen species (ROS), including oxygen ions, free radicals, and lipid hydroperoxides, are augmented. Although cellular antioxidants such as glutathione are

capable of neutralizing ROS and preventing cellular damage, depletion of antioxidants leads to ROS accumulation which can result in cellular dysfunction and apoptosis (Sordillo and Aitken, 2009).

The protein NFE2L2 is a transcription factor regulating genes that encode proteins involved in response to production of free radicals. In basal conditions, NFE2L2 interacts with KEAP1 protein, which targets NFE2L2 to degradation. In response to oxidative stress, KEAP1 dissociates from NFE2L2 in the cytoplasm, thereby allowing NFE2L2 to translocate to the nucleus and initiate transcription of a cascade of antioxidant response genes (Townsend et al., 2003; Kaminskyy and Zhivotovsky, 2014). Although *NFE2L2* expression was not altered by FA + BCKA supplementation, the significant reduction in expression of *KEAP1* in FA + BCKA treated PLEHs suggests an enhanced antioxidant response, which agrees with a previous report that dietary Ile supplementation improved antioxidant capacity by decreasing intestinal *KEAP1* expression (Zhao et al., 2014).

Glutathione reductase (*GSR*) is an enzyme that regenerates GSH from GSSG as a part of the GSH redox cycle. GSH protects cells from excessive amounts of ROS through a reduction reaction, where GSH will be converted to the oxidized version of itself, GSSH. The enzyme *GSR* plays a role in reducing GSSH back to GSH so the cycle can begin to have antioxidant effects again (Townsend et al., 2003). The decrease in *GSR* expression in the FA + BCKA treatment suggest that there is a decreased need for GSR to be used converting GSSG to its antioxidant counterpart GSH. Although GSH was not measured as a part of this experiment, we are conjecturing that decreased GSR is indicative of GSH at an adequate level to meet antioxidant balance in the FA + BCKA treatment.

Reducing ROS production should alleviate cellular oxidative damage. Oxidative phosphorylation is essential for the generation of ATP but generates substantial amount of ROS, especially when fatty acid oxidation is enhanced. Mitochondrial transporter protein UCPs create proton leaks across the inner mitochondrial membrane, thus uncoupling oxidative phosphorylation from ATP synthesis. As a result, protonmotive force decreases slightly and attenuates mitochondrial ROS production (Brand, 2000). Therefore, the greater expression of *UCP2* in FA + BCKA treated PLEHs suggests a potentially lower ROS production from enhanced proton leak across the inner mitochondrial membrane. This is also supported by a lower mitochondria membrane potential which is a measure of the protonmotive force (Crowley et al., 2016; Zorova et al., 2018). Expression of *UCP2* may also alter oxidative substrates within the mitochondria. Evidence suggests that increasing UCP2 alters mitochondrial catabolism by preferentially promoting mitochondrial fatty acid oxidation rather than the oxidation of glycolytic derived pyruvate (Pecqueur et al., 2008). This preferential use of fatty acid instead of pyruvate in the FA + BCKA treatment due to elevated *UCP2* expression may also be a route of decreasing intracellular TAG compared to the FA.

Apoptosis is defined as programmed cell death characterized by nuclear fragmentation, chromatin condensation, and cellular shrinkage. It can occur as a normal result of development and aging but can also occur as a cellular response to disease damage or noxious agents such as ROS. The primary apoptotic pathway occurs through the activation of caspases such as *CASP3* and *CASP7* (Aizawa et al., 2020). With lower expression of *CASP3* and *CASP7* in FA + BCKA treated PLEHs, there is likely less destruction of cellular structure (Li et al., 2014). In human liver disease, high rates of apoptosis are associated with decrease liver function (Wang et al., 2016) and progression of FL to more advanced liver disease (Luedde et al., 2014). Signaling for apoptosis

can result from ROS imbalance, for example *NF-Kb* allows for a cytoprotective pathway that up-regulates antiapoptotic genes including caspase inhibitors (Tang et al., 2001). Inhibition of this *NF-Kb* pathway can increase oxidative stress (Luedde et al., 2014). Although *NF-Kb* was not altered by FA + BCKA supplementation, decreased expression of *KEAP1, GSR* and increase expression of *UCP2* suggest an improved ability to neutralize ROS and decreased ROS generation.

When evaluating results within this experiment, it is important to note the limitations of the model used. Isolating PLEH from more individual lactating cows will offer more biological replicates to support the power of the PLEH model. However, isolating viable hepatocytes can be a challenge and the model has demonstrated viable PLEH (Zhou et al., 2018). Concentrations of plasma nutrients and hormones may also vary based on the location of plasma sampling. The nutrient concentrations of the CM is based on circulating levels collected from the coccygeal vein on postpartum d 4 (Zhou et al., 2016b). Use of nutrient and hormone concentrations within the portal vein or hepatic artery may be more accurate in replicating the hepatocyte environment but collection of such data is more invasive (Reynolds et al., 2003). The current PLEH model evaluating intracellular TAG accumulation, gene expression, and mitochondrial membrane potential should be replicated with analysis of other phenotypic data to support these conclusions.

CONCLUSION

Fatty liver was modeled using cultured primary bovine liver cells enriched with hepatocytes exposed to a customized media intended to mimic the circulating plasma of early lactation dairy cows susceptible to the disease. Within this model, FA + BCKA decreased intracellular TAG accumulation and altered expression of genes regulating lipid metabolism, antioxidant response, and apoptosis. We suggest that FA + BCKA decrease hepatic TAG accumulation and improve liver function when exposed to FAs by reducing fatty acid uptake,

enhancing fatty acid oxidation and export, enhancing antioxidant responses and reducing ROS production. *In vivo* data are required to confirm these finding.

APPENDICES

APPENDIX A

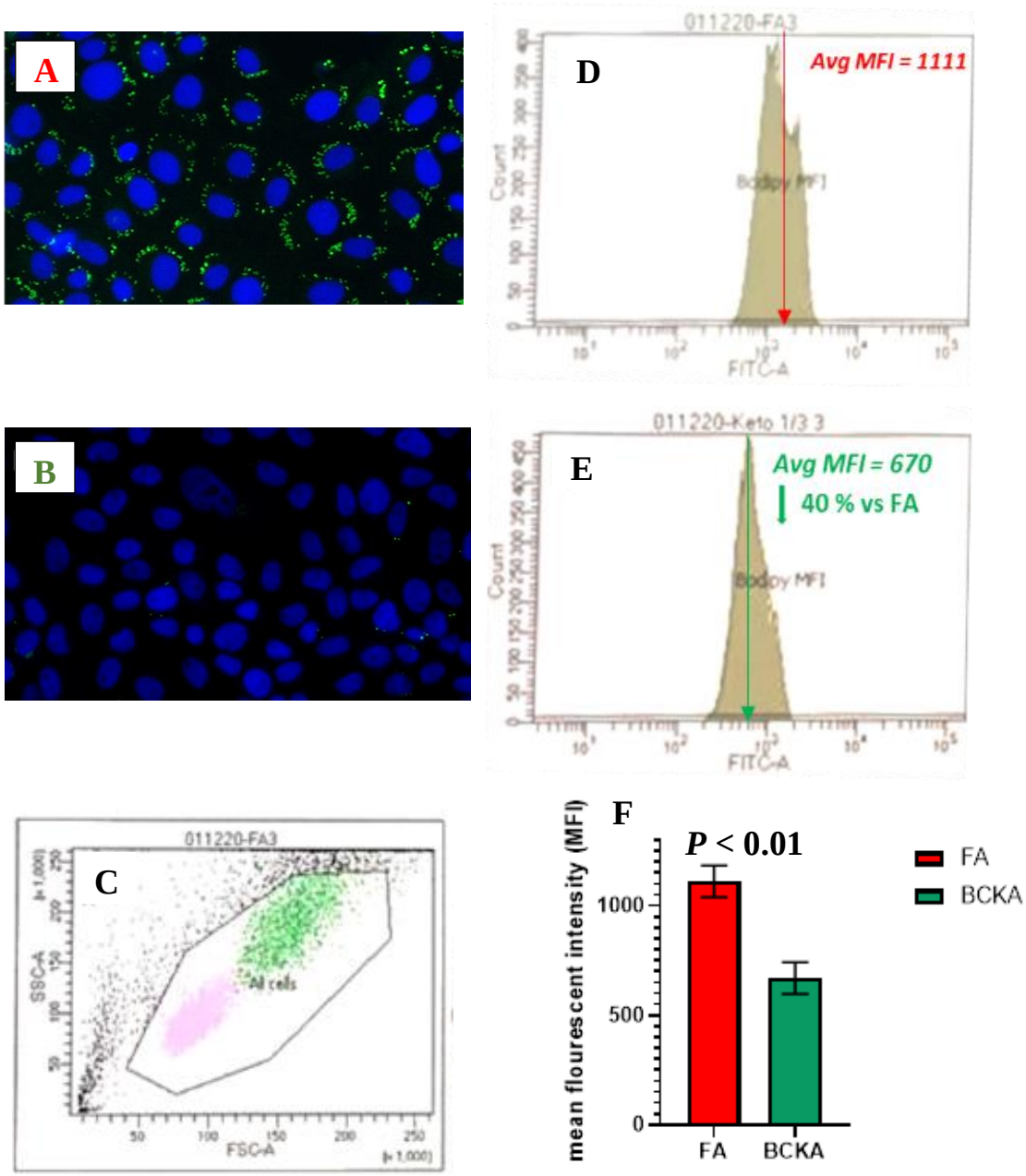
TABLES AND FIGURES

Table 2.1. Nutrient composition of customized media for PLEH[1]

Parameter	Customized Media[2]
Amino Acids (mg/L)	
Alanine	16.74
Arginine	10.95
Asparagine	5.17
Aspartic Acid	0.47
Cysteine	1.77
Glutamic Acid	4.69
Glutamine	41.13
Glycine	36.52
Histidine	12.38
Isoleucine	13.85
Leucine	21.98
Lysine	11.85
Methionine	3.49
Phenylalanine	8.71
Proline	8.08
Serine	10.23
Threonine	10.00
Tryptophan	3.74
Tyrosine	10.55
Valine	27.51
Other (mg/L)	
Albumin	3350
Choline	0.52
Glucose	621.13
Insulin	0.0004
Fatty Acids, mM	
Palmitic Acid (16:0)	0.23
Stearic Acid (18:0)	0.23
Oleic Acid (18:1)	0.20
Linoleic Acid (18:2)	0.29
Arachidonic Acid (20:4)	0.05

[1]primary bovine liver cells enriched with hepatocytes
[2]customized media based on early lactation circulating plasma concentrations from coccygeal vein (Zhou et al., 2016b)

Figure 2.1. Intracellular TAG[1] accumulation of PLEH[2] exposed to FA or BCKA[3] treatment for 72 h. Intracellular TAG is stained with Bodipy 493/503 (green) and nucleus with Hoechst (blue). Intracellular TAG observed for FA (A) and BCKA (B) under fluorescent microscope. Recognition of PLEH (C) and quantification of TAG in FA (D) and BCKA (E) with flow cytometry (F).



[1]triacylglyerol
[2]primary bovine liver cells enriched with hepatocytes
[3]customized medium + 1mM fatty acid cocktail (FA), FA + BCKAs at 33% equivalent of corresponding plasma BCAAs on d 4 postpartum (FA + BCKA)

Table 2.2. Expression of genes related to fatty acid metabolism in PLEH[1] treated with FA or BCKA[2]

	Treatment			
Genes (AU)[3]	FA	FA + BCKA	SEM	*P*-value
FA uptake				
CD36	0.697	0.378	0.249	0.38
FABP4	0.098	0.054	0.058	0.59
FATP5	0.459	0.351	0.034	0.07
TAG export				
MTTP	0.373	0.284	0.191	0.74
β-oxidation				
PPARA	0.409	0.281	0.056	0.14
ACOX1	1.10	1.11	0.033	0.80
ECHS1	0.177	0.231	0.013	0.03
HADH	0.647	0.713	0.068	0.50

[1]primary bovine liver cells enriched with hepatocytes
[2]customized medium + 1mM fatty acid cocktail (FA), FA + BCKAs at 33% equivalent of corresponding plasma BCAAs on d 4 postpartum (FA + BCKA)
[3]arbitrary units

Table 2.3. Mitochondria membrane potential, expression of genes related to antioxidant response, and apoptosis in PLEH[1] with FA or BCKA[2]

	Treatment			
Genes (AU)[3]	FA	FA + BCKA	SEM	*P*-value
Antioxidant response				
GCLC	0.856	0.754	0.053	0.20
GSR	16.2	6.56	2.18	0.02
KEAP1	0.943	0.712	0.043	0.01
NFE2L2	0.936	0.888	0.028	0.25
Membrane Potential				
UCP2	0.345	0.442	0.022	0.02
ΔΨ (RFU[4])	2274	1900	289	< 0.01
Apoptosis				
CASP3	0.757	0.545	0.057	0.04
CASP7	0.374	0.208	0.054	0.07

[1]primary bovine liver cells enriched with hepatocytes
[2]customized medium + 1mM fatty acid cocktail (FA), FA + BCKAs at 33% equivalent of corresponding plasma BCAAs on d 4 postpartum (FA + BCKA)
[3]arbitrary units
[4]mitochondrial membrane potential dyed with tetramethylrhodamine ethyl ester and quantified with flow cytometry relative fluorescent units (RFU)

APPENDIX B

SUPPLEMENTAL MATERIALS

Figure S.1. Plasma valine, leucine, and isoleucine concentrations on d4 postpartum cows with high (50.8 mg/g) vs low (7.8 mg/g) liver TAG content

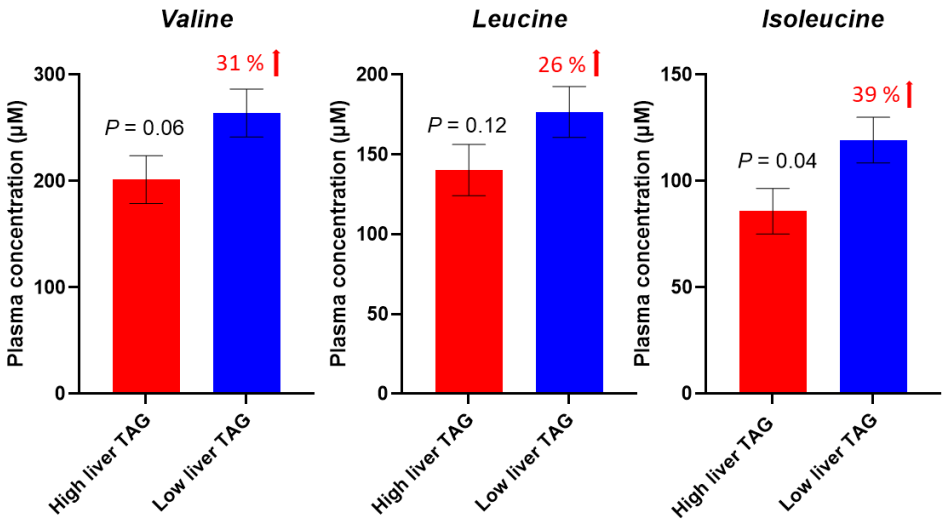


Table S.1. List of primers used for RT-qPCR

Gene	F or R[1]	Sequence (5' – 3')
ACOX1	F	TTG-TGG-CGG-ACA-TGG-CTA-TT
	R	TGA-GTG-CAC-CTG-GTC-GTA-AC
BCKDHA	F	TCA-GCG-GAG-TTC-ATA-GAC
	R	CTT-GTA-GAA-TTT-GAG-CAC-CTT
BCKDHB	F	AGT-AAC-AAG-TGC-CTT-AGA-TAA-C
	R	CTG-CGA-TTC-CAA-TTC-CAA
Beta Actin	F	GCC-CTG-AGG-CTC-TCT-TCC-A
	R	GCG-GAT-GTC-GAC-GTC-ACA
CASP3	F	ACA-GAA-CTG-GAC-TGT-GGT-ATT-G
	R	GAA-GTC-TGC-CTC-AAC-TGG-TAT-T
CASP7	F	CAT-AGT-GAG-GCA-GAG-AAG-GAA-C
	R	ATC-GCT-GAA-CAC-AGG-GAA-TAG
CDO1	F	ATG-GAA-GCC-TAT-GAG-AGC-AA
	R	TTC-AGA-AAG-CAG-TGG-GAG-TC
CD36	F	GAC-GGA-TGT-ACA-GCG-GTG-AT
	R	GAA-AAA-GTG-CAA-GGC-CAC-CA
DBT	F	CAT-AAC-ATA-CAA-GGC-TTC-TCA-T
	R	TTC-AAT-TCA-GTG-GCA-ATC-TC
DLD	F	AAT-GTT-GGT-TGT-ATT-CCT-TCT-AA
	R	TCT-GCT-CCA-TCA-TCT-TCT-C
ECHS1	F	CTG-CTG-TCA-ATG-GCT-ATG
	R	GAT-GGT-TCC-TAT-TAG-AAT-CTC-C
FABP4	F	GTT-CGA-TGC-AAA-CGT-CAT-CCA
	R	AGA-TGG-TGC-TGG-AAT-GTG-TCA
FATP5	F	GCT-TGT-CCT-TGG-AGT-CCT-CAG-T
	R	GCC-GAC-AGT-CAT-CCC-AGA-AG
GAPDH	F	GAA-GGT-CGG-AGT-GAA-CGG-AT
	R	TTC-TCT-GCC-TTG-ACT-GTG-CC
GCLC	F	TAC-GAT-CAG-TTG-GCT-ACC
	R	CCG-AGT-TCT-ATC-ATC-TAC-AGA
GCN2	F	GAC-TTC-AGA-CCT-CCC-TTG-CC
	R	GCC-TGT-TCA-TCA-GCA-TCC-CA
GSR	F	GAG-AAC-GCT-GGC-ATT-GAG
	R	AGC-AGG-CAG-TCA-ACA-TCT
HADH	F	CAA-TGA-CCA-GCC-AGA-AGA
	R	CAG-AAG-ACG-GTT-CAC-GAT-A
HIBCH	F	GTT-CTC-AAC-TAA-TAC-TGT-GGA-A
	R	ATA-CTT-CTG-GCA-AGG-TCT-T
IVD	F	CAC-TCC-AAC-CTC-TGT-ATC-A
	R	TCC-GAT-GTA-TTC-ACC-ACT-T
KEAP1	F	ACA-CCA-CGG-TGT-TCC-AAT-CA
	R	GGC-TCT-CGT-TGG-ACA-TTC-CT
MCCC1	F	TAC-GGC-AAG-GAG-ATG-AAG
	R	CAG-GCT-AAG-GAG-GAA-GTC

Table S.1. (cont'd)

Gene	F or R[1]	Sequence (5' – 3')
MCCC2	F	GAG-ACA-CAT-TAG-TTA-CAG-GAT-T
	R	GAA-GGA-AGA-GCA-GAG-GAA
MTTP	F	AAT-GGT-CCG-TCA-AGT-TCT
	R	GAA-TGT-CAA-GGC-TGT-AAG-TAG
NFE2L2	F	CCT-CAA-AGC-ACC-GTC-CTC-AG
	R	GCT-CAT-GCT-CCT-TCT-GTC-GT
NFκB	F	ACC-TCC-TTC-CGC-AAA-CTC-AG
	R	ATA-GGT-CCT-TCC-TGC-CCG-TA
PPARA	F	GGC-AGA-ACC-AGA-GCA-CAG-AT
	R	CCG-GCC-ACA-GAC-TGT-TAC-TT
RPS9	F	GAA-CAA-ACG-TGA-GGT-CTG-GAG-G
	R	TTA-CCT-TCG-AAC-AGA-CGC-CG
SLC1A5	F	CTG-CTC-ATT-CGC-TTC-TTC
	R	TTC-TCC-ATC--TCC-ACA-ATC-TT
SLC3A2	F	AAT-CCG-TCT-TCA-TTC-TTA-GC
	R	CTG-GAG-TGT-GAC-AGG-TAA
SLC7A5	F	GCT-CTA-TGC-CTT-CTC-CAA
	R	GCC-AGA-TGA-ACC-TTG-ATG
UCP2	F	ATG-CCA-TTG-TCA-ACT-GTG
	R	GGT-CGT-CTG-TCA-TTA-GGT

[1]forward (F) or reverse (R) transcription

Amino Acid Metabolism

Amino acid signaling

Increased expression of general control nonderepressible 2 (*GCN2*) indicates that the FA + BCKA treatment reduced leucine signaling (Table S.1). Expression of *GCN2* is significantly downregulated in the FA + BCKA treatment compared to FA ($P = 0.01$). The gene *GCN2* is highly expressed in the liver during AA starvation (Sood et al., 2000). The significant decrease of *GCN2* in the FA + BCKA treatment is indicative of AA availability for the PLEH. With no differences in AA concentration between treatments, it is likely that the FA + BCKA treatment interacts with a feedback loop relating to BCAA metabolism (Harper et al., 1984). The decrease expression of *GCN2* may influence the phenotypic results of decreased TAG content in the FA + BCKA treatment.

There is evidence that activity of *GCN2* will stimulate response for other redox homeostasis pathways, such as *NRF2* and *KEAP1* expressed pathways (Sood et al., 2000; Yuan et al., 2021). In a murine model, *GCN2* knockout animals fed a high fat diet had reduced symptoms of liver dysfunction, insulin resistance, and hepatic steatosis compared to wild type mice fed a high fat diet (Liu et al., 2017b). Reducing expression of *GCN2* is indicative of AA availability to the PLEH while also reducing effects of FL.

Amino acid transport

There is significant upregulation in expression of solute carrier family 3 member 2 (*SLC3A2)* in the FA + BCKA treatment ($P < 0.01$). There is no change in the expression of other AA transport related genes solute carrier family 1 member 5 (*SLC1A5)* and solute carrier family 7 member 5 (*SLC7A5, P* > 0.12).

The subunit SLC3A2 is involved with the import of EAA, and has high specificity for Leu (Nachef et al., 2021). An increase in expression of this gene indicates that even without an increase in supplementation of AA, the supplementation of FA + BCKA is enough to increase uptake capacity for some AA transporters while also potentially effecting cell signaling. For example, increasing intracellular influx of Leu can activate mTOR which effects many cellular processes such as protein synthesis (Zhenyukh et al., 2017; Nachef et al., 2021).

Amino acid catabolism

When evaluating expression of genes relating to AA catabolism, there is a significant downregulation of branched chain keto acid dehydrogenase subunit α (*BCKDHA)* and branched chain keto acid dehydrogenase subunit β (*BCKDHB)* in the FA + BCKA treatment ($P < 0.01$). Expression of dihydrolipoamide dehydrogenase (*DLD*)

decreases in the FA + BCKA treatment compared to the CON ($P = 0.07$). There is no change in the expression of cysteine dioxygenase type 1 (*CDO1),* dihydrolipoamide branched chain transacylase *(DBT),* 3-hydroxyisobutyryl-CoA hydrolase *(HIBCH),* isovaleryl-CoA dehydrogenase *(IVD),* methylcrotonyl-CoA carboxylase subunit 1 *(MCC1),* or methylcrotonyl-CoA carboxylase subunit 2 *(MCC2)* between treatments ($P > 0.13$).

The downregulation of 3 subunits of the BCKDH complex, *BCKDHα, BCKDHβ,* and *DLD* indicates that there is reduced expression of BCKDH due to FA + BCKA. The BCKDH complex is used as the secondary and rate limiting step of BCAA catabolism, catalyzing the oxidative carboxylation of BCKA. BCKA and its end products act as competitive inhibitors for BCKDH activation (Harper et al., 1984). This indicates that hepatocytes were successfully treated and delivered with FA + BCKA as there is likely increased competitive inhibition to express BCKDH subunits.

Table S.2. Expression of genes related to amino acid signaling in PLEH[1] treated with FA or BCKA[2]

AA signaling	Treatment			
Gene (AU)[3]	FA	FA + BCKA	SEM	*P*-value
GCN2	0.948	0.745	0.039	0.01

[1]primary bovine liver cells enriched with hepatocytes
[2]customized medium + 1mM fatty acid cocktail (FA), FA + BCKAs at 33% equivalent of corresponding plasma BCAAs on d 4 postpartum (FA + BCKA)
[3]arbitrary units

Table S.3.Expression of genes related to amino acid membrane transport in PLEH[1] treated with FA or BCKA[2]

AA transport	Treatment			
Gene (AU)[3]	FA	FA + BCKA	SEM	*P*-value
SLC1A5	0.485	0.604	0.047	0.12
SLC3A2	0.699	0.872	0.021	< 0.01
SLC7A5	0.450	0.546	0.039	0.13

[1]primary bovine liver cells enriched with hepatocytes
[2]customized medium + 1mM fatty acid cocktail (FA), FA + BCKAs at 33% equivalent of corresponding plasma BCAAs on d 4 postpartum (FA + BCKA)
[3]arbitrary units

Table S.4. Expression of genes related to amino acid catabolism in PLEH[1] treated with FA or BCKA[2]

Amino acid catabolism	Treatment			
Gene (AU)[3]	FA	FA + BCKA	SEM	*P*-value
BCKDHA	0.872	0.667	0.032	< 0.01
BCKDHB	4.44	1.75	0.397	< 0.01
CDO1	1.14	0.826	0.267	0.44
DBT	0.578	0.614	0.614	0.74
DLD	1.48	0.978	0.158	0.07
HIBCH	0.627	0.910	0.152	0.24
IVD	0.636	0.569	0.034	0.19
MCCC1	0.662	0.716	0.037	0.34
MCCC2	0.634	0.934	0.126	0.13

[1]primary bovine liver cells enriched with hepatocytes
[2]customized medium + 1mM fatty acid cocktail (FA), FA + BCKAs at 33% equivalent of corresponding plasma BCAAs on d 4 postpartum (FA + BCKA)
[3]arbitrary units

CHAPTER 3

ABOMASAL INFUSION OF BRANCHED-CHAIN AMINO ACIDS OR BRANCHED-CHAIN KETO-ACIDS ALTER MILK PRODUCTION AND LIVER TRIGLYCERIDES IN EARLY LACTATION DAIRY COWS

ABSTRACT

Dairy cows are at high risk of fatty liver disease in early lactation due to a negative energy and protein balance. Current measures to increase milk and component yield while also preventing fatty liver are not always effective. Our objective was to determine the impact of branched-chain amino acids (BCAA) and their corresponding ketoacids (branched-chain ketoacids, BCKA) on production performance and liver triglyceride accumulation of Holstein cows in the first 3 wk postpartum. Thirty-six multiparous Holstein cows were used in a randomized block design experiment. Cows were abomasally infused for the first 21 d postpartum with solutions of 1) saline (**CON**, n = 12); 2) **BCAA** (n = 12) including 67 g valine, 50 g leucine, and 34 g isoleucine; and 3) **BCKA** (n = 12) including 77 g ketovaline, 57 g ketoleucine, and 39 g ketoisoleucine. All cows received the same diet. Treatment effects were determined using PROC GLIMMIX in SAS. No differences ($P > 0.22$) were detected for body weight, body condition score and dry matter intake. Cows receiving BCKA had significantly lower liver triglyceride % compared to CON (6.60 vs 4.77 %, SEM = 0.49). Infusion of BCAA increased milk yield (39.5 vs 35.3 kg/d, SEM = 1.80), milk fat yield (2.10 vs 1.69 kg/d; SEM = 0.08), lactose yield (2.11 vs 1.67 kg/d; SEM = 0.07), and solids yield (2.47 vs 2.00 kg/d; SEM = 0.21) compared with CON ($P \leq 0.05$). Compared with CON, cows receiving BCAA had lower glucose (55.0 vs 59.2 mg/dL, SEM = 0.86, $P < 0.01$) but higher BHBA (9.17 vs 6.00 mg/dL, SEM = 0.80, $P < 0.01$) in blood. Overall, results indicate that BCAA supplementation improves milk production while BCKA supplementation reduced TAG accumulation in the liver in early lactation cows.

INTRODUCTION

Over 50% of all dairy cows develop fatty liver in early lactation, costing the U.S. dairy industry over $60 million annually in lost milk production, cow health, and profitability (Bobe et al., 2004). Fatty liver disease (**FL**) in dairy cows is marked by accumulation of triglycerides (**TAG**) within hepatocytes resulting in compromised liver function. Higher liver TAG content usually leads to more compromised liver function and lactation performance (Bobe et al., 2004; Petta et al., 2016; Geng et al., 2021). Although various preventative strategies have been developed, no strategy consistently reduces liver TAG content (Piepenbrink and Overton, 2003; Bobe et al., 2004).

There is a negative association between circulating branched chain amino acids (**BCAA**) and hepatic accumulation of triacylglycerol (**TAG**) (Lobley, 1992). Supplementing BCAA in monogastric models has demonstrated properties such as improved insulin sensitivity and protein synthesis to alleviate liver disease (Arakawa et al., 2011; Tajiri and Shimizu, 2018), The causal relationship between BCAA metabolism and fatty liver has not been explored in the ruminant, likely due to the seminal study in the field demonstrating that BCAAs have the lowest rate of removal by liver under various nutritional and physiological conditions (Heitmann and Bergman, 1980). However, a BCAA metabolite branched chain keto-acid (**BCKA**) that can be taken up in the ruminant liver (Harper et al., 1984) decreased intracellular TAG accumulation and altered lipid metabolism in primary bovine hepatocytes (Gallagher et al., 2021). Therefore, BCAA supplementation may be able to reduce liver TAG accumulation via BCKAs. In agreement with this, a recent study demonstrated that supplementing rumen protected BCAA in addition to propylene glycol significantly decreased liver TAG accumulation (Leal Yepes et al., 2019).

In addition, increasing MP through post ruminal supply of essential amino acids (**AA**) can influence milk production characteristics such as increased milk and component yield (Rulquin et al., 1993; Lean et al., 2018). Branched chain amino acids are nonessential AA that make up 20.8% of total AA mass in milk (NASEM, 2021). Supplementing early lactation dairy cows with BCAA has shown to increase metabolizable protein (Leal Yepes et al., 2019) while Leu and Ile increase milk and milk component yield in mid-lactation cows (Yoder et al., 2020). Additionally, BCAA can impact AA signaling pathways, such as through the activation of mammalian target of rapamycin (**mTOR**) and influence protein and nutrient partitioning within the mammary gland (Nie et al., 2018; Yoder et al., 2020)

Therefore, we hypothesized that abomasal infusions of BCAA or BCKA will improve lactation performance and decrease liver TAG accumulation during early lactation. The objective was to determine if abomasal infusion of BCAA or BCKA in the first 21 d postpartum effects intake, milk yield and components, plasma nutrients, and liver TAG content of Holstein cows**.**

MATERIALS AND METHODS

Experimental Design and Dietary Treatments

All procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (protocol no. PROTO202000206). The experiment was conducted as a randomized block design with multiparous (lactation ≥ 2) Holstein dairy cows (n = 38) from the Michigan State University Dairy Cattle Teaching and Research Center (Lansing, MI) between November 2021 and March 2022. A total of 38 cows were blocked according to the expected calving date into 3 blocks (Block 1, n=11: enrolled 11/14/21-12/25/21; Block 2, n=13: enrolled 12/28/21-02/05/22; Block 3, n=14: enrolled 01/31/22-03/14/22) Cows within block were balanced for parity, and previous lactation 305 mature equivalent milk yield. All cows enrolled underwent

a rumenotomy procedure prepartum (46.1 ± 2.2 d before expected calving date) unless previously cannulated (n = 6) for abomasal infusion. Within each block, cows were randomly assigned to 1 of 3 treatments: control (**CON,** n = 12) receiving daily abomasal saline infusion without branched-chain amino acids or ketoacids; branched-chain amino acid (**BCAA,** n = 13) receiving daily abomasal infusion of 67 g valine, 50 g leucine, and 34 g isoleucine; and branched-chain ketoacid (**BCKA**, n=13) receiving daily abomasal infusion of 77 g ketovaline, 57 g ketoleucine, and 39 g ketoisoleucine. Dosage of BCAA and BCKA was based on a preliminary block of 6 cows (data not presented) that successfully increased plasma BCAA concentration by approximately 30%. The daily dose of BCAAs and corresponding BCKAs were matched on a 1:1 molar ratio across BCAA and BCKA treatments. The BCAA components are comprised of > 99.9 % purity L-leucine, L-isoleucine, and L-valine (ACP Chemicals, Montreal, QB). The BCKA components are comprised of the following calcium salts: α ketoleucine, α ketoisoleucine, α ketovaline, (> 99% purity, Stru Chem, Wujiang City, China). Calcium chloride dihydrate (> 98% purity, Fischer Chemical), was added to CON and BCAA infusate to match the Ca levels in BCKA treatment. Treatments were delivered to cows daily via continuous abomasal infusion using a peristaltic pump (Spires et al., 1975; Westreicher-Kristen and Susenbeth, 2017). Seven liters of aqueous solution were administered to each cow daily at an adjusted pH of 2.5 with HCl and NaOH to mimic naturally occurring abomasal pH Treatment solutions were prepared and administered to cows no more than 3 days after preparation. Calving day was considered d 0. Initiation of abomasal infusion for all animals occurred at 1200 h (± 15 min) on d 1 after calving. Abomasal infusions were halted during milking for approximately 3 – 4 h/d. Abomasal lines were flushed with 120 mL water after halting and before continuing infusion to ensure treatment delivery to abomasum.

Animal Management

Animals were housed in individual sawdust-bedded tie stalls and were milked 3 times per day at approximately 0630, 1430, and 2230 h. Animals were fed ad libitum at 115% of expected intake. All animals were fed a common TMR diet (Table 3.1) over the course of the study and received the same close-up dry period diet prior to enrollment. Diets were formulated in AMTS Farm Cattle (Agricultural Modeling and Training Systems, LLC; Groton, NY) to meet nutrient requirements for lactating cows at 14 DIM with an expected milk yield of 36.3 kg/d, milk fat 3.7%, and milk protein 3.0%. Animals were fed once daily at 0930 h, weekly samples were analyzed via wet chemistry with the means reported (Table 3.2).

Feed and Milk Sample Collection and Analyses

Throughout the study, TMR samples were collected weekly, frozen at −20°C, and composited monthly for wet chemistry analysis (Cumberland Valley Analytical Services, Waynesboro, PA https://www.foragelab.com/Services/Forage-and-Feed/Lab-Procedures/). The values for NE_L, RUP, RDP, and amino acids, were predicted using software of the Nutrient Requirements of Dairy Cattle (v.8, The National Academies of Sciences, Engineering, and Medicine) based on ingredient composition, with the observed mean DMI, BW, milk yield, and milk components for each treatment (Table 3.3 and Table 3.4). As fed intake was recorded daily as the as fed(kg) offered subtracting as fed(kg) orts the following day. Early lactation TMR was collected once per week and dried at 100°C for 24 h in a forced-air oven to determine dry matter (**DM**). Each weekly DM was used for the following 7 days to calculate dry matter intake (**DMI**). Daily DMI (kg) was calculated as the as fed intake (kg) × DM.

Body condition score (**BCS**) was measured by 3 trained investigators on a 5-point scale in 0.25-increments (Wildman et al., 1982). Body weight (**BW**) and BCS were measured once weekly

on d 1, 7, 14, 21 ± 1 prior to liver biopsy collection. Dry matter intake as a % of BW (**DMI, % BW**) was calculated daily using the weekly BW for each individual cow. Milk yield was recorded 3×/d over the course of the study. Milk was sampled for 3 consecutive milkings 2×/week. Milk samples were stored in a sealed tube with preservative (Bronopol tablet; D&F Control Systems, San Ramon, CA) and stored at 4°C until analyzed. Infrared spectroscopy was used to analyze and report milk fat (%), true protein (%), lactose (%), solids (%), and milk urea nitrogen (**MUN**) (mg/dL) and flow cytometry used to analyze somatic cell count (**SCC**) 1000/mL (Central Star Cooperative, Inc; Grand Ledge, MI). Daily milk yield was summed over 3 milk shifts on the same calendar date. Weekly milk component concentrations and yield were calculated as the 2 daily weighted average collected per week, weighted by the corresponding milk shift yield, and averaged together by week. Fat-corrected milk (**FCM**) was calculated as [(0.4324 × milk yield kg) + (16.216 × fat yield kg)] and energy-corrected milk (**ECM**) was calculated as [(12.82 ×fat yield kg) + (7.13 × protein yield kg) + (0.323 × milk yield kg) according to Hutjens, (2005).

Blood Sample Collection and Analysis

Blood was sampled from the coccygeal vein on 0 (actual calving day before treatment), 3, 7, 14, and 21 d relative to parturition between 0700 – 0830 h before TMR delivery and after cows returned from milking parlor. Venipuncture blood was collected using a 21-gauge × 1.5 cm into evacuated tubes containing 158 USP sodium heparin (Becton Dickinson and Company, Franklin Lakes, NJ) for plasma separation. All blood samples were placed on ice and centrifuged at 2000 × *g* for 20 min at 4°C. Plasma was aliquoted and stored at -20°C until analysis.

Blood samples were analyzed for plasma glucose, non-esterified fatty acid (**NEFA**), and β-hydroxybutyrate (**BHB**). An enzymatic calorimetric method assay was used to quantify glucose, NEFA and BHB in plasma via commercially available kits according to manufacturer's

instructions (Glucose; Sigma – Aldrich, St. Louis, MO; NEFA: Wako USA, Mountain View, CA; BHB: EFK Diagnostics-Stanbio, United States). Absorbance was measured with a micro-plate reader (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA).

Liver Sample Collection and TAG content Quantification

Liver was sampled via puncture biopsy (Dann et al., 2005; Zhou et al., 2016b) and was collected on all cows enrolled on study on d 1, 7, 14, 21 ± 1. On biopsy sampling days, animals were escorted from morning milking to animal handling chutes before having access to feed. Location of biopsy was determined with ultrasound of hepatic tissue and the area was anesthetized with 10 mL of 2% lidocaine (Vet One, Boise, ID). Biopsy samples were harvested and immediately frozen and stored in liquid N until further analysis. Approximately 50 mg of liver samples was used for liver TAG quantification using the method by Zhou et al. (2016).

Statistical Analysis

All data were analyzed using PROC Glimmix of SAS v9.4 (SAS Institute, Cary, NC) with the model: $Y = \mu + b_i + T_j + D_k + TD_{jk} + A_{l:ij} + e_{ijklm}$ where Y = the dependent, continuous variable, μ = overall mean, b_i = fixed effect of block, T_i = fixed effect of treatment, D_k = fixed effect of time, $A_{l:ij}$ = random effect of the l th cow nested within block × Treatment and e_{ijklm} = the residual error. Fixed effect of time is the day or week relative to parturition in which samples were collected for each variable. Parity (second vs. third lactation and greater) and previous 305-d milk yield were kept in the model as covariate for all variables when significant ($P < 0.05$). The blood and liver samples collected prior to administration of treatments were also included as covariate for NEFA, BHB, and liver TAG content respectively if found to be significant. Blood metabolites and liver TAG were analyzed at various time points that were not equally spaced with a heterogeneous variance over time. Therefore, the first order ante-dependence covariance structure ANTE (1),

spatial power covariance structure SP(POW), or Toeplitz covariance structure (TOEP) was chosen based on akaike's information criterion values for repeated measures of liver TAG and blood metabolites. For equally spaced data, autoregressive covariance structure AR (1) was used for repeated measures. Somatic cell count was log transformed for statistical analysis and back transformed for presentation. Effects were post hoc Tukey adjusted and significant statistical differences were declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

RESULTS

Health

Health-related problems occurred during the experiment and causes for the removal of cows are summarized in Table 3.5. A total of 1 BCAA and 1 BCKA cows developed peritonitis following treatment enrollment at the rumenotomy surgery site and were removed from the study following the recommendation of attending veterinarians from the Michigan State University department of Large Animal Clinical Sciences. Data from the cows removed were excluded from statistical analysis.

DMI, Body Weight and Body Condition Score

As expected, DMI (kg) and DMI, % of BW of cows in all treatments (Figure 3.1A) gradually increased ($P < 0.01$) from calving to 21 d. However, DMI did not differ among treatment groups when reported in kg or % of BW ($P > 0.18$, Table 3.6). The BW, BCS, weekly BW change, and weekly BCS change were not affected by BCAA or BCKA treatments ($P > 0.58$, Table 3.6).

Milk Production and Composition

Overall yield of milk (Figure 1B), FCM and ECM (Table 3.7, Figure 3.1D-E) were greater in BCAA-supplemented cows than in CON cows ($P \leq 0.05$). Compared with CON, BCAA infusion increased milk fat, lactose, and solids yield (Figure 3.2, $P \leq 0.05$) and tended to increase protein

yield (Figure 3.2, $P = 0.10$). The percent of fat, lactose, solids in milk, as well as SCC did not differ between treatments (Table 3.7, $P \geq 0.28$) and protein % was lower in BCAA cows compared to CON (Figure 3.1A, $P = 0.01$). Similarly, the significantly higher milk:DMI in BCAA- cows (Figure 3.1C) was also driven by greater milk yield compared with CON cows as DMI did not differ between CON and BCAA cows (18.9 vs 19.2 kg/d). Compared with CON, MUN was also significantly higher in the BCAA treatment (P = 0.02, Table 3.7, Figure 3.2F).

Blood Biomarkers and Liver TAG Content

Plasma glucose, NEFA, and BHBA, and liver TAG content are reported in Table 3.8. Cows receiving BCAA had lower plasma glucose ($P < 0.01$) and higher plasma BHBA ($P < 0.02$) compared to both CON and BCKA. Plasma NEFA concentration in BCAA cows was not different from CON cows but higher ($P = 0.03$) than in BCKA cows (Figure 3.3B). Although plasma concentrations of NEFA, BHBA, and glucose in BCKA cows were not different from CON cows, BCKA supplementation reduced liver TAG compared to CON but not compared to BCAA cows (Figure 3.3D, BCAA vs BCKA, $P = 0.12$; BCAA vs CON, $P = 0.80$; BCKA vs CON, $P = 0.03$).

DISCUSSION

Ingredient, Nutrient Composition, and NASEM Evaluation of Diets

Dairy cows are in negative MP balance due to the abrupt increase in MP demand and inability of cows to consume sufficient protein during early lactation (Bell et al., 2000). Abomasal supplementation of AA increases the amount of protein that is directly available to the abomasum as MP. Other studies have shown that increase supplementation of individual AA increases total MP supply (Osorio et al., 2013; Zhou et al., 2016b). Nutrient composition evaluation of diets using the NASEM (2021) model revealed impacts of BCAA and BCKA infusion on MP (Table 3.3 and Table 3.4). When evaluating numerical differences, in RUP, the BCAA treatment has higher RUP

as a % of DM and in total g supplied to the cows Consequently, NE_L-allowable milk for BCAA treatment was 29.7 kg/d compared to 26.4 kg/d for BCKA treatment and 26.9 kg/d for CON, which partially explained the significantly higher milk yield (39.5 kg/d) in BCAA cows compared to CON (35.3 kg/d) and BCKA (35.1 kg/d) cows during early lactation.

In addition to MP and RUP, BCAA infusion also successfully altered dietary AA % of MP as well and the g of each AA provided through dietary means. According to the NASEM (2021) model, BCAA infusion elevated Val, Leu, Ile as a % of MP and as dietary AA (g). Due to the elevations of Val, Leu, Ile in the BCAA treatment, the proportion of other AA as a % of MP are lower than the CON and BCKA treatment. For example, Met which has been supplemented in dairy diets in past studies (Batistel et al., 2017; Zhou et al., 2018), made up a lower % of MP although the mass of dietary Met is similar between treatments. This model uses a factorial, rather than proportional approach, to assess the essential AA needed to fulfill a metabolic function. Due to the variable efficiency of AA use for different metabolic purposes, the profile of AA being metabolized to lactating cows is likely more influential on metabolism than the additive effect of metabolized AA (NASEM, 2021). Evaluating both the available MP and AA profile of these treatments within a model demonstrates that the BCAA treatment increases MP and altered the AA profile through Val, Leu, Ile. Increasing RUP and altering the EAA profile of the BCAA treatment indicate more AA to mitigate the effects of negative energy balance (**NEB**) and negative protein balance (**NPB**).

As building blocks of protein, supplementation of EAA may also increase milk protein synthesis. Using the NASEM (2021) model, BCAA appearance in milk protein were estimated to be 68 g Ile, 116 g Leu, and 76 g in CON cows. In BCAA supplemented cows, 79 g Ile, 135 g Leu, and 88 g of Val were estimated to appear in milk using the same model, which account for 11 g of

Ile (32%), 19 g of Leu (38%) and 12 g of Val (18%) of the BCAAs supplied (34 g Ile, 50 g Leu, and 67 g of Val) via abomasal infusion.

Effects on DMI, BW, and BCS

Although there were numerical differences to the total MP supplied, the weekly change in both BW and BCS were equally negative in all treatments. This decrease in BW and BCS in early lactation is normal in dairy cows as they typically are unable to eat enough to meet nutrient demands of milk production and therefore experience NEB and NPB (Bauman and Currie, 1980).

There were no differences in DMI among treatments, which follows previously reported DMI result from Leal Yepes et al (2019) and Yoder et al (2020). The Leal Yepes et al (2019) evaluated BCAA and BCAA supplementation with propylene glycol drench in the early lactation while Yoder et al (2020) evaluated jugular infusion of Leu and Ile in the mid lactation. Although the dosages and lactation period differed to this experiment, the BCAA did not affect DMI. In an early lactation study by Leal Yepes et al (2019), multiparous Holsteins blocked to receive a top-dress supplement as a control, BCAA, or BCAA with an additional drench of propylene glycol from parturition to 35 days in milk. Yoder et al (2020)also evaluated the effects of specific BCAA, Leu and Ile on mid lactation dairy cows through jugular infusions. Although DMI gradually increased from calving to 21 d as expected, this gradual increase in DMI apparently was not sufficient to meet the demand of milk yield as indicated by the loss of BW and BCS throughout the course of this study. Consequently, the cows in this study had decreased circulating glucose and elevated NEFA and BHBA levels in blood.

Milk Production and Composition

Branched chain amino acids stimulate protein synthesis by activating mTOR signaling pathway (Lynch and Adams, 2014; Yoon, 2016). For dairy cows, BCAAs are expected to improve

total milk production by enhancing milk protein synthesis, but the effect of BCAA supplementation on lactation performance has been inconsistent. Yoder et al. (2020) found that mammary plasma flow and milk yield increased in response to jugular infusion of Leu at 50 g/d and Ile at 22 g/d, which is likely due to a greater mammary anabolic activity through stimulation of mTOR (Cant et al., 2018). The mTOR pathway is a central regulator for mammalian cell growth that is an AA sensor and plays a role in AA balance, biosynthesis, and degradation. BCAAs can play a modulatory role in stimulating the protein kinase B pathway resulting in mTOR stimulation and further metabolic activity (Nie et al., 2018). In contrast, a significant increase in milk yield was not observed in Leal Yepes et al (2021) rumen protected supplementation of 101.1 g/d Leu, 40.8 g/d Ile, and 61 g/d Val in the early lactation. Inconsistencies of BCAA effect on milk production could be due to differences in BCAA dosage and method of delivery between different experiments.

Similar to the increased milk and component yield reported by Yoder et al. (2020), we also observed increased yield of milk, ECM, FCM, milk fat, lactose, and milk solids and a tendency towards higher protein yield in response to BCAA infusion. With the % of milk fat, lactose, and solids not different among treatments, such increase in yield originates from a 4.2 kg/d increase in milk yield from BCAA infusion. It is also noteworthy that despite a significantly lower protein % in cows receiving BCAA infusion, protein yield tended to be higher in response to BCAA infusion compared with CON, suggesting that at our infusion dose, BCAA potentially also lead to greater mammary anabolic activity through stimulation of mTOR and or additional MP.

Although BCKA were abomasally infused at equal molar amounts to BCAA, BCKA supplementation failed to induce changes in milk yield, milk component yield or composition. Alternatively, a study evaluating the supplementation of keto-Leu from Vandehaar et al (1988)

reported that supplementing rumen soluble Na bound keto-Leu to dairy cows at 0.75% of DM increased milk fat % and total fat and FCM yield. This discrepancy between our milk fat results and Vandehaar et al (1988) may be due the addition of keto-Ile and keto-Val in our BCKA treatment. It is also worthy to note that rumen soluble BCKAs have similar structure to branched chain FA found in the rumen, which can alter the rumen microbial environment and increase milk fat synthesis (Papas et al., 1984; Liu et al., 2018). Milk component effects of ruminally feeding the BCKA treatment remains to be investigated.

The Milk:DMI ratio is an estimate of feed efficiency but does not account for the individual BW and BCS change in individual cows. This ratio is limited as it does not measure endogenous tissue turn over or accumulation, however, changes in this ratio may still be able to give us a gross estimate of feed efficiency. Hence, the higher Milk:DMI ratio suggest a more efficient conversion of intake to milk in BCAA supplemented cows, which is largely due to a 4.2 kg/d increase in milk yield while maintaining a similar DMI compared to CON.

The BCAA treatment was the only treatment supplemented with additional amine groups and resulted in elevated MUN, suggesting additional N secretion in BCAA treatment compared to the CON and BCKA treatments. Similarly, the Leal Yepes et al (2019) trial supplementing early lactation cows with rumen protected BCAA also reported elevated MUN values compared to the control. The BCKA treatment did not increase MUN compared to the CON as BCKA have the ability to be re-aminated to BCAA when tissues are in a BCAA deficit and have Gln available. This means that BCKA may decrease N excretion due to its decreased nitrogen load and ability to deaminate (Holeček, 2020).

Glucose, BHBA, and NEFA

Glucose is the main fuel source for the mammary gland and is used throughout the body as an energy substrate. Most glucose production of ruminants is sourced from gluconeogenesis occurring in the liver from ruminal derived propionate (Bauman and Currie, 1980). As the precursor for lactose production in the mammary gland, glucose is taken up by the mammary gland (Annison and Linzell, 1964; Lin et al., 2016). In our experiment, BCAA infusion decreased plasma glucose concentration, but increased lactose yield compared with CON. This is in contrast to other early lactation ruminant and monogastric studies where BCAA supplementation either did not change or increased plasma glucose concentration (Leal Yepes et al., 2021; Rezaei et al., 2022). Whether the decrease we reported in plasma glucose concentration in the BCAA treatment was due to increased mammary uptake or compromised gluconeogenesis in the liver requires further investigation. In consideration for the increased mammary uptake theory, other trials have reported Leu and Ile increase mammary plasma flow (Yoder et al., 2020) which would increase glucose delivery to the mammary gland as mammary plasma flow is correlated with milk yield (Prosser et al., 1996).

Plasma NEFA concentration is used as an index for adipose mobilization after parturition. It is estimated that half of NEFA entering the plasma are oxidized or incorporated into milk triglycerides (Bell, 1995). With similar BW and BCS change in this experiment across treatments, similar plasma NEFA were expected for all treatments. The increased NEFA concentration in the BCAA treatment does not parallel with the BW and BCS response but may relate to increased milk fat yield resulting from NEFA incorporation into milk lipids. However, more evidence will be required to support this notion (e.g. milk FA profile).

The ketone body BHBA can originate from incomplete FA oxidation in the liver. The early lactation period is typically marked with elevated plasma BHBA due to tissue mobilization to support milk synthesis (Drackley, 1999). Beta-hydroxybutyrate can be beneficial to the animal in a NEB as it serves as a water soluble energy source that can be delivered to different tissues (McArt et al., 2013). Excessive plasma levels of BHBA are typically associated with decreased milk production and potential health consequences, such as increased incidence of hyperketonemia and FL disease and decreasing β-oxidation, TCA activity, and gluconeogenesis in hepatocytes (Bobe et al., 2004; McArt et al., 2013). In contrast to Leal Yepes et al (2019), reporting no change in BHBA in response to BCAA supplementation, plasma BHBA concentration was significantly higher in BCAA infused cows compared with CON in this study. Such increase was likely due to enhanced hepatic FA oxidation in BCAA cows to satisfy a higher energy demand for elevated milk production and components compared to the other treatments. It is also noteworthy that BHBA concentrations in the BCAA cows of this study averaged 9.17 mg/dL (0.88 mmol/L); thus our cows were not in a state of subclinical (1.2-2.9 mmol/L) or clinical (>3.0 mmol/L) ketosis (Duffield et al., 2009).

Liver Triglyceride Content

Based on liver TAG content, fatty liver disease in dairy cattle can be categorized as a % of liver TAG wet weight into mild (1-5%), moderate (5-10% and severe FL (>10%) (Bobe et al., 2004). Both mild and moderate FL are at risk of developing severe symptoms and pathogenesis. Milk production, health and reproductive status can decrease weeks after liver TAG returns to < 1% (Veenhuizen et al., 1991). Therefore, strategies that effectively reduce liver TAG content can minimize negative impact of FL disease. Previous studies have demonstrated that BCAA supplementation in a murine model decrease liver TAG (Arakawa et al., 2011). Similarly, in dairy

cattle, supplementation of BCAA in addition to propylene glycol in the Leal Yepes et al (2021) experiment also observed a decrease in liver TAG in the early lactation period. Propylene glycol drenching can be used in the prepartum and postpartum period to increase plasma glucose, but it not an effective FL preventative strategy as it not effective as a TMR supplement (Grummer, 2008). Such decrease in liver TAG content of the BCKA treatment is likely due to alterations in metabolic profiles and nutrient partitioning across tissue types relating glucose and lipid metabolism by BCAA (Yoon, 2016; Tajiri and Shimizu, 2018). Despite improved lactation performance, liver TAG content was not significantly reduced by BCAA supplementation. In contrast, BCKA did not alter lactation performance, but the significant reduction in liver TAG content placed BCKA cows in the mild FL category with an average liver TAG content of 4.77%. With the same molar amount infused, multiple factors may contribute to the discrepancy of BCAA and BCKA impact on liver TAG content. First, the reduced liver TAG content in response to BCKA infusion is likely due to alterations in liver lipid metabolism as treating bovine hepatocytes with BCKA decreased expression of genes related to lipid uptake while increasing the expression of lipid oxidation genes (Gallagher et al., 2021). With no net BCAA uptake by the liver, BCAAs supplemented need to be converted to BCKAs in extra-hepatic tissues before reaching the liver to impact hepatic metabolism (Harper et al., 1984; Webb et al., 2019). Although equal molar amount of BCAA and BCKA were supplemented, not all BCAAs were likely converted to BCKA. Rather than getting converted to BCKAs, mammary gland and other tissue may take up BCAAs for milk and tissue protein synthesis, further reducing amount of BCAA capable of impacting liver metabolism. Further analysis of hepatic metabolism and circulating AA, ketoacids, and other metabolites are required to uncover the underlying mechanism for such discrepancy.

CONCLUSIONS

Abomasal infusion of BCAA in the first 3 wk of lactation increased yield of milk and milk components, while decreasing plasma glucose, and increasing plasma BHBA and NEFA. The BCAA treatment did not affect liver TAG content % of wet weight or DMI. Supplementation of BCKA in early lactation effectively decreased liver TAG accumulation but did not alter lactation performance, plasma metabolites or DMI. The BCAA treatment had numerically higher predicted RUP and MP while also altered the predicted AA profile delivered to the cows. Abomasal infusion of BCAA and BCKA modulated different outcomes in different tissue types. Further understanding of how these nutrients effect liver and mammary metabolic mechanisms should be pursued.

APPENDIX

TABLES AND FIGURES

Table 3.1. Ingredient composition of common diet fed during early lactation

Ingredient	% of DM
Conventional Corn Silage	20.8
Ground Corn	17.4
Alfalfa Silage	15.4
BMR Corn Silage	14.0
Soybean Meal	10.1
Cottonseed, Whole	8.43
Protein Mix	6.18
Amino Plus[1]	2.44
Caledonia Pass[2]	1.14
Sodium Sesquinate Refined	0.976
Calcium Carbonate	0.793
Ground Corn	0.602
Urea	0.163
Smartamine M[3]	0.0683
Alfalfa Hay	5.62
Mineral Vitamin Mix	1.97
Ground Corn	0.434
MIN – AD[4]	0.403
Calcium Carbonate	0.394
Calcium Phosphate Dical	0.376
Salt	0.197
Sodium Sesquinate refined	0.0896
Selenium 06	0.0393
Bleachable fancy tallow	0.0138
Intellibond VITAL 5[5]	0.0104
Vitamin E	0.00674
Vitamin A	0.000843
Vit D3 500[6]	0.000281

[1]Ag Processing Inc., Omaha, NE
[2]Caledonia Farmers Elevator, Caledonia, MI
[3]Adisseo, Alpharetta, GA
[4]MIN-AD Inc., Winnemucca, NV
[5]Micronutrients USA LLC, Indianapolis, IN
[6]Baltivet, Dubingai, Lithuania

Table 3.2. Analyzed composition of the common diet fed to multiparous Holstein cow supplemented with BCAA or BCKA in the early lactation period

Measured Component (% DM)[1]	TMR
NE_L (Mcal/kg of DM)	0.70
CP	17.5
aNDFom[2]	30.6
ADF	20.9
Lignin	11.6
Starch	25.7
Crude Fat	4.3
Ash	7.7
Ca (% of DM)	1.2
P (% of DM)	0.4
Mg (% of DM)	0.3
K (% of DM)	1.3
Na (% of DM)	0.4
Lys:Met	2.69
Met	0.29
Lys	0.78
Leu	1.33
Ile	0.68
Val	0.86

[1]means reported from weekly TMR samples composited and do not include abomasal treatments. (Cumberland Valley Analytical Services, Waynesboro, PA).
[2]amylase-treated NDF.

Table 3.3. Predicted nutrient components using reported animal inputs in multiparous Holstein cow supplemented with BCAA or BCKA in the early lactation period

Predicted Composition[1]	Treatments[2]		
	CON	BCAA	BCKA
NE_L (Mcal/kg of DM)	1.77	1.77	1.77
CP (% of DM)	17.6	18.3	17.6
RDP (% of DM)	12.0	11.9	12.0
RUP (% of DM)	5.6	6.3	5.6
Dig RUP (% of DM)	4.6	5.2	4.6
RDP supplied (g/d)	2274	2280	2441
RUP supplied (g/d)	1061	1207	1139
MP supplied (g/d)	1964	2099	2092
MP from Microbial CP (g/d)	1110	1120	1170
MP from RUP (g/d)	870	1000	930
aNDFom (% of DM)	30.5	30.3	30.5
ADF (% of DM)	20.6	20.4	20.6
Starch (% of DM)	26.3	26.1	26.3
Total FA (% of DM)	4.55	4.52	4.55
NE_L allowable milk (kg/d)	21.95	22.69	23.90
MP allowable milk (kg/d)	26.91	29.67	26.39
Ca (% of DM)	1.07	1.06	1.07
P (% of DM)	0.41	0.40	0.41
Mg (% of DM)	0.29	0.28	0.29
Cl (% of DM)	0.28	0.27	0.28
K (% of DM)	1.22	1.21	1.22
Na (% of DM)	0.43	0.43	0.43
S (% of DM)	0.19	0.19	0.19

[1]Predicted by Nutrient Requirements of Dairy Cattle (v.8, The National Academies of Sciences, Engineering, and Medicine) based on ingredient composition, with the observed mean DMI, BW, milk yield, and milk components for each treatment.
[2]CON = control (saline 0.9%); BCAA = branched chain amino acids (67 g valine, 50 g leucine, and 34 g isoleucine); BCKA= branched chain keto-acids (77 g keto-valine, 57 g keto-leucine, and 39 g keto-isoleucine)

Table 3.4. Predicted amino acid composition using measured animal inputs[1] in multiparous Holstein cows supplemented with BCAA or BCKA[2] in the early lactation period

Amino Acid	Dietary AA (% of MP)[3]			Dietary AA (g)[4]		
	CON	BCAA	BCKA	CON	BCAA	BCKA
Met	2.50	2.36	2.52	49.1	49.6	52.7
Lys	8.84	8.35	8.90	173.6	175.3	186.3
Leu	14.07	15.80	14.16	276.3	331.6	296.6
Ile	7.66	9.02	7.72	150.4	189.4	161.6
Val	8.82	11.80	8.88	173.3	247.6	186.0
Arg	10.33	9.78	10.40	202.9	205.2	217.8
His	3.37	4.14	4.40	85.8	86.8	92.1
Phe	8.55	8.09	8.61	168.0	169.8	180.3
Thr	6.48	6.13	6.52	127.2	128.6	136.5
Trp	2.10	2.00	2.11	41.2	41.6	44.2

[1]Predicted by Nutrient Requirements of Dairy Cattle (v.8, The National Academies of Sciences, Engineering, and Medicine) based on ingredient composition, with the observed mean DMI, BW, milk yield, and milk components for each treatment.
[2]CON = control (saline 0.9%); BCAA = branched chain amino acids (67 g valine, 50 g leucine, and 34 g isoleucine); BCKA = branched chain keto-acids (77 g keto-valine, 57 g keto-leucine, and 39 g keto-isoleucine)
[3]percent of total MP that that individual AA supplied from diet and treatment
[4]mass of each AA modeled from diet and treatment

Table 3.5. Frequency of health incidence in multiparous Holstein cow supplemented with BCAA or BCKA in the early lactation period

	Treatment[1]		
Variable	CON	BCAA	BCKA
Cows[2]	12	13	13
Ketosis[3]	1	2	1
Hypocalcemia[4]	2	2	1
Metritis	0	0	0
Displaced abomasum	0	0	0
Mastitis	0	0	0
Excluded Cows[5]	0	1	1
Peritonitis	0	1	1

[1]CON = control (saline 0.9%); BCAA = branched chain amino acids (67 g valine, 50 g leucine, and 34 g isoleucine); BCKA = branched chain keto-acids (77 g keto-valine, 57 g keto-leucine, and 39 g keto-isoleucine)

[2] n cows enrolled on each treatment

[3]as defined by ≥ moderate urinary ketones Ketostix (Bayer AG, USA) and treated with oral propylene glycol

[4]defined by recumbency and or muscle tremors received oral bolus of Bovikalc (Boehringer Ingelheim, USA)

[5] n cows excluded from study and analysis due to unrecoverable disease as defined by veterinarians

Table 3.6. Dry matter intake, body weight, and body condition score of multiparous Holstein cows supplemented with BCAA or BCKA in the early lactation period

Parameter	Treatments[1]				*P* -Value		
	CON	BCAA	BCKA	SEM	Trt	Time	Trt ×Time
DMI, kg/d	18.9[a]	19.2[a]	20.4[a]	0.61	0.22	< 0.01	0.40
DMI, % BW	2.95[a]	2.90[a]	3.11[a]	0.12	0.18	< 0.01	0.42
BW, kg	644[a]	644[a]	660[a]	13.0	0.58	< 0.01	0.95
BCS	3.16[a]	3.12[a]	3.12[a]	0.08	0.92	< 0.01	0.73
BW change, kg/wk	-15.6[a]	-17.0[a]	-14.4[a]	3.4	0.86	0.03	0.88
BCS change, unit/wk	-0.10[a]	-0.13[a]	-0.09[a]	0.04	0.69	0.26	0.46

[1] CON = control (saline 0.9%); BCAA = branched chain amino acids (67 g valine, 50 g leucine, and 34 g isoleucine); BCKA = branched chain keto-acids (77 g keto-valine, 57 g keto-leucine, and 39 g keto-isoleucine)

[2]Tukey difference in means indicated with different superscript

Table 3.7. Milk yield and milk components for multiparous Holstein cow supplemented with BCAA or BCKA in the early lactation period

	Treatments[1,2]				*P* -Value		
	CON	BCAA	BCKA	SEM	Trt	Time	Trt × Time
Yield (kg/d)							
Milk	35.3[b]	39.5[a]	35.1[b]	1.80	0.02	< 0.01	0.34
Fat	1.69[b]	2.10[a]	1.67[b]	0.08	0.02	0.02	0.72
Protein	1.25[a]	1.46[a]	1.30[a]	0.07	0.10	0.17	0.80
Lactose	1.67[b]	2.11[a]	1.69[b]	0.07	0.02	< 0.01	0.49
Solids	2.00[b]	2.47[a]	2.02[b]	0.21	0.05	< 0.01	0.15
FCM[3]	41.1[b]	52.0[a]	42.0[a,b]	3.3	0.04	< 0.01	0.50
ECM[4]	40.8[a]	50.8[a]	41.8[a]	3.1	0.05	< 0.01	0.53
Components (%)							
Fat	4.93[a]	4.90[a]	4.84[a]	0.13	0.85	< 0.01	0.87
Protein	3.73[a,b]	3.51[b]	3.83[a]	0.08	0.01	< 0.01	0.85
Lactose	4.88[a]	4.86[a]	4.83[a]	0.02	0.28	< 0.01	0.26
Solids	5.74[a]	5.70[a]	5.70[a]	0.03	0.51	< 0.01	0.11
MUN[5](mg/dL)	11.5[a,b]	12.7[a]	11.2[b]	0.40	0.02	0.11	0.78
SCC[6]	1.55[a]	1.52[a]	1.64[a]	0.07	0.46	< 0.01	0.42
Milk:DMI	1.90[b]	2.11[a]	1.79[b]	0.08	0.03	< 0.01	0.23

[1]CON = control (saline 0.9%); BCAA = branched chain amino acids (67 g valine, 50 g leucine, and 34 g isoleucine); BCKA = branched chain keto-acids (77 g keto-valine, 57 g keto-leucine, and 39 g keto-isoleucine)

[2]Tukey difference in means indicated with different superscript

[3]fat corrected milk calculated as FCM, kg = (0.4324 × Milk yield, kg) + (16.216 × Fat yield, kg) (Hutjens, 2005).

[4]energy corrected milk calculated as ECM, kg = (12.82 × fat yield, kg) + (7.13 × protein yield, kg) + (0.323 × milk yield, kg) (Hutjens, 2005).

[5]milk urea nitrogen

[6]somatic cell count log transformed for normality

Table 3.8. Plasma concentrations of metabolites and liver TAG concentration for multiparous Holstein cow supplemented with BCAA or BCKA in the early lactation period

Parameters	Treatments[1,2]				*P* -Value		
	CON	BCAA	BCKA	SEM	Trt	Day	Trt × Day
Glucose, mg/dL	59.2[a]	55.0[b]	60.3[a]	0.86	< 0.01	0.29	0.58
BHBA, mg/dL[3]	6.00[b]	9.17[a]	5.83[b]	0.80	< 0.01	0.06	0.11
NEFA, mEq/L[4]	705[a,b]	806[a]	552[b]	66.8	0.03	< 0.01	0.76
Liver triglycerides, %[5]	6.60[a]	6.15[a,b]	4.77[b]	0.49	0.03	0.28	0.74

[1]CON = control (saline 0.9%); BCAA = branched chain amino acids (67 g valine, 50 g leucine, and 34 g isoleucine); BCKA = branched chain keto-acids (77 g keto-valine, 57 g keto-leucine, and 39 g keto-isoleucine)
[2]Tukey difference in means indicated with different superscript
[3]betahydroxybutyrate concentration
[4]nonesterified fatty acids concentration
[5]liver TAG reported as % wet tissue weight

Figure 3.1. Dry matter intake (A), milk yield (B), milk:DMI (C), fat corrected milk (D) and energy corrected milk (E) recorded over time for multiparous Holstein cow supplemented with BCAA or BCKA in the early lactation period

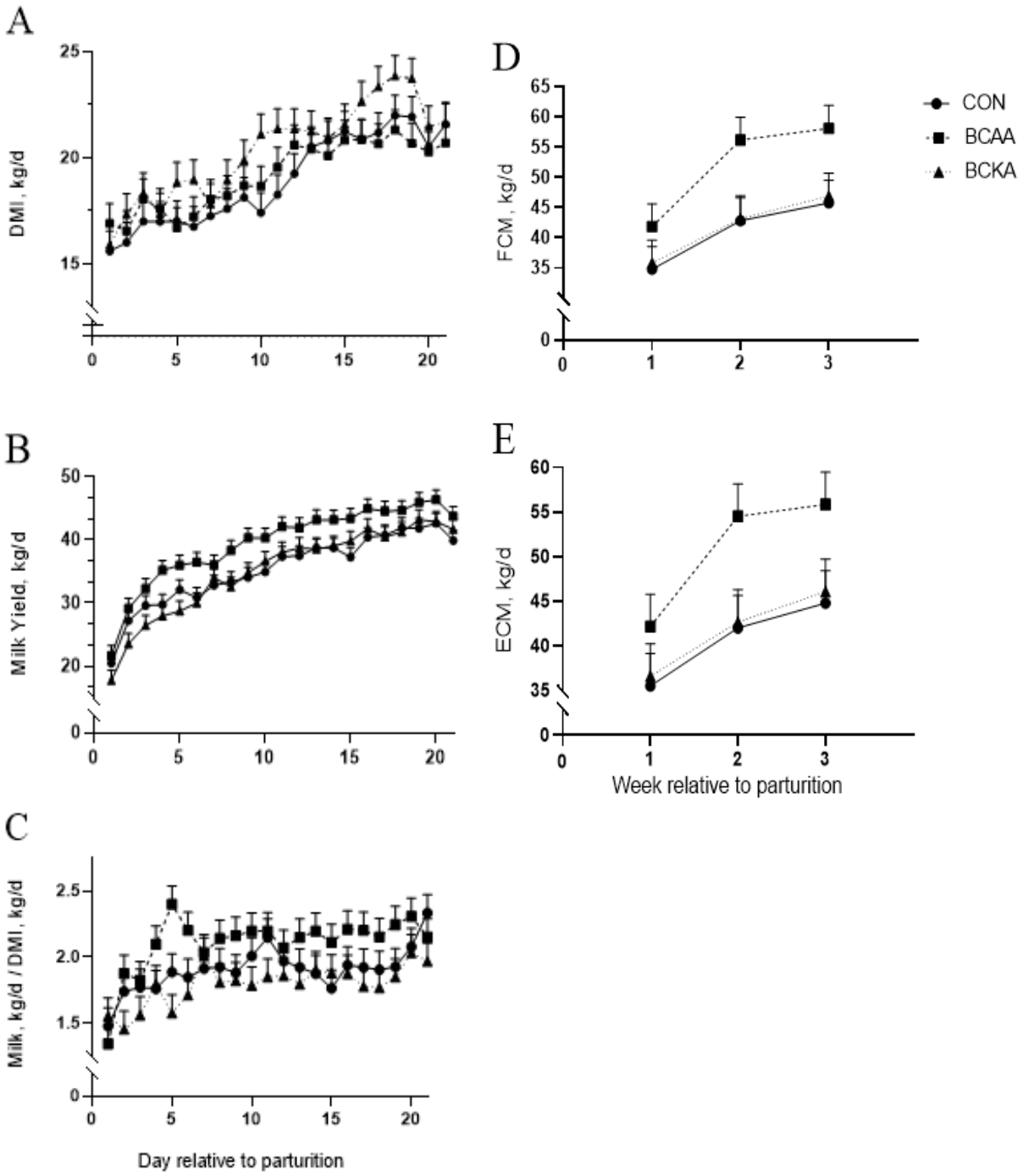


Figure 3.2. Milk components reported over week relative to parturition for multiparous Holstein cow supplemented with BCAA or BCKA in the early lactation period

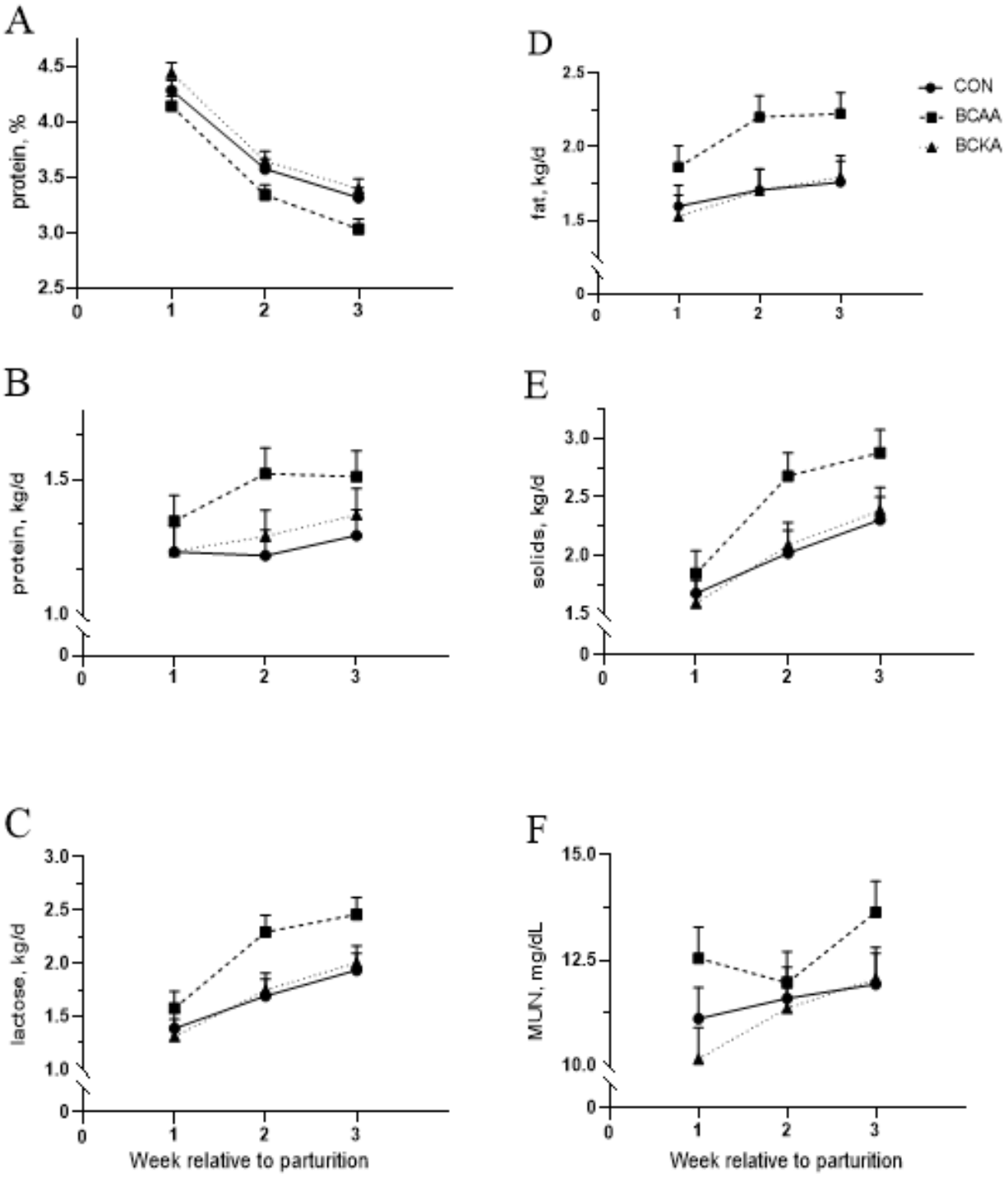
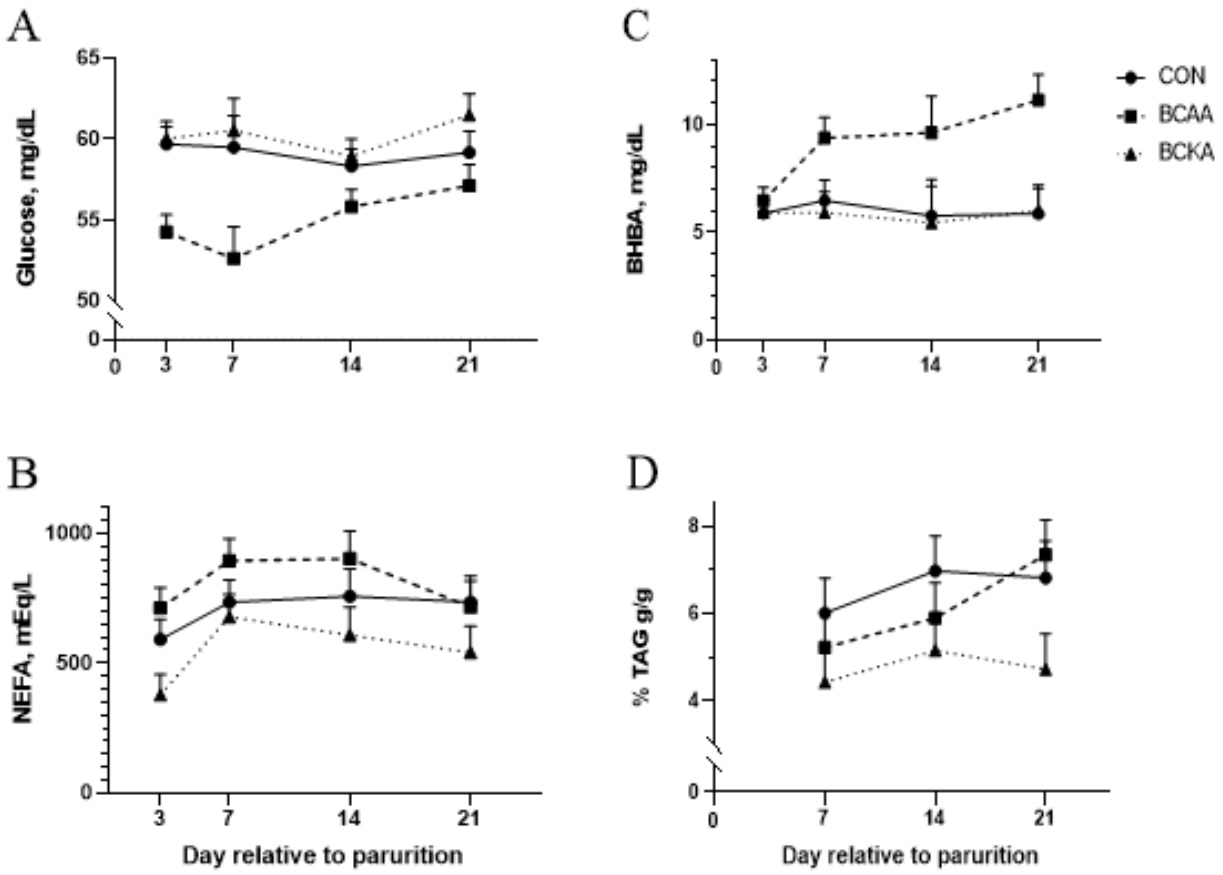


Figure 3.3. Plasma metabolites and liver TAG reported over sample collection day for multiparous Holstein cow supplemented with BCAA or BCKA in the early lactation period



CHAPTER 4

IMPLICATIONS AND CONCLUSIONS

IMPLICATIONS AND CONCLUSIONS

Current nutritional interventions fail to prevent FL in lactating dairy cows (Grummer, 1993; Cooke et al., 2007). This metabolic disease is very costly for the U.S. dairy industry as it effects milk production, incidence of disease, and reduces reproductive efficiency within herds (Bobe et al., 2004). Understanding the metabolic implications and pathogenesis of FL can promote strategies aimed at preventing FL in dairy cows. By evaluating the mechanistic response of FL and lactation to BCAA and BCKA, we can better promote nutrition strategies.

This thesis aimed to evaluate how BCAA or BCKA interact with ruminant metabolism in two different models mimicking early lactation. The first study evaluated the effect of FA + BCKA on TAG accumulation and metabolism in primary bovine liver cells enriched with hepatocytes within an early lactation circulating environment. The FA + BCKA media decreased intracellular accumulation of TAG by 40%. This response is likely due to altered expression of genes related to lipid metabolism, including decreased intracellular FA uptake, increased antioxidant capacity, and decreased apoptosis. Considering the benefits of BCAA on liver disease in monogastrics, such as altering inulin sensitivity and FA oxidation (Arakawa et al., 2011; Tajiri and Shimizu, 2018), the metabolite BCKA likely influences similar metabolic pathways. Not all genes expression measured was influenced by FA + BCKA. Including measurements of TAG and gene expression at other timepoints would also help our understanding of when and how FA + BCKA reduces intracellular TAG. To further understand how FA + BCKA interacts with lipid metabolism, western blotting of enzymes and measuring rate of oxidation within PLEH should be completed. The efficacy of FA + BCKA to decrease intracellular TAG accumulation *in vitro* indicates that this pathway should be evaluated *in vivo* to understand physiological effects in dairy cows.

To continue understanding the outcomes of study 1, a second study analyzed how abomasal infusions of BCAA or BCKA effect production parameters and liver TAG accumulation in early lactation dairy cows. The BCAA infusion improved milk yield by 9% while also increasing milk component yield and altering plasma metabolites but did not alter liver TAG % of wet weight. Abomasal infusion of BCKA decreased liver accumulation of TAG % of wet weight by 28% compared to the control but did not affect production or plasma metabolite outcomes. Considering circulating NEFA level starts to increase in late gestation, BCKA supplementation prepartum may also be helpful in reducing the risk of FL in dairy cows.

The BCAA likely influences nutrient signaling and modulation pathways due to alterations in the profile of EAA being supplied to the cow. Other studies have indicated that mTOR activation within the mammary gland could be responsible for increased mammary activity leading to elevated milk and component yields (Yoder et al., 2020). Branched chain keto-acids significantly decreased hepatic TAG accumulation in both the *in vitro* and *in vivo* model, but further analysis of gene expression and mitochondrial function of liver tissue is necessary to determine the mechanism by which this occurs *in vivo.* Although a negative association between BCAA and FL are documented (Lobley, 1992), equal molar amounts of BCAA supplementation failed to decrease liver TAG in this study. Such discrepancy is likely due to post-absorptive mechanism of mammary uptake of BCAA and hepatic uptake of BCKA. Future research should evaluate plasma concentrations of AA and ketoacids, liver, muscle, and adipose tissue BCAA/BCKA metabolism to understand effects on cow liver health and function. Continued data monitoring after supplementation of BCKA and BCAA ceased would be insightful to determine the long-term health and production outcomes of the infused supplements.

These studies conclude that further research should determine if supplementing BCKA is an economically viable nutrition strategy to prevent FL disease. Both study 1 and 2 were designed to deliver the BCKA at 33% of circulating BCAA concentrations. A dosage response on lactating cows to BCKA should be evaluated to determine the rate at which BCKA is most effective. Continued evaluation of BCAA on lactation response should proceed to improve efficiency of dairy production. Through these studies, we conclude that BCKA can decrease susceptibility to FL, while BCAA promotes production of milk and milk components.

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