Determining the Role of Energy on Mammary Gland Utilization of Plasma Amino Acids

By: Amara D. Benn

Abstract

Our overall objective is to investigate nutritional strategies that upregulate signaling pathways of milk protein synthesis directing nitrogen towards milk production, therefore reducing the amount of nitrogen waste entering the environment. Targeting the mTORC1 pathway shows promise as it coordinates nutrient and environmental cues to stimulate cellular anabolic processes, like protein and fat synthesis. Previously, our lab has demonstrated that insulin and amino acids (AA) synergistically stimulate mammary mTORC1 activity. We hypothesized that glucogenic energy, by stimulating insulin secretion and potentiating AA activation of mammary mTORC1 activity, would increase sequestration of AA for milk protein synthesis. To test our hypothesis, 36 peak-lactation Holstein cows were used in a 4x4 repeated Latin square design with four 28-d periods and four treatments arranged as a 2x2 factorial. The two treatment factors were energy source: glucogenic (GE) or ketogenic (KE) and total metabolizable AA: 10% deficient (DAA) or sufficient (BAA). Blood samples from a subset of 20 cows were collected from coccygeal vessel (artery) and subcutaneous abdominal (mammary) vein 6 timepoints during the last 2 days of each period. Plasma was isolated by centrifugation and mixed with isotopically labeled AA as internal standards for AA analysis by liquid chromatography-mass spectrometry. Mammary blood flow was calculated based on the Fick principle, using Phe+Tyr as non-metabolizable markers. Plasma concentrations, mammary extraction efficiency and uptake were calculated, and statistical analysis was applied. Plasma amino acid concentration, extraction and uptake were significantly affected by energy source and AA level, with few treatment interactions. Although the ketogenic diet had an increased response in milk protein g/d ($P = 0.03$), overall increased sequestration of amino acids was not observed, with only significant increases in Arg ($P = 0.01$) and Ser ($P = 0.02$) mammary uptake.

Milk Production

A recent meta-analysis has shown global food demand will increase between 35% – 65% over the period 2010-2050 (van Dijk et al., 2021). Increasing the availability of high quality, nutritious food is essential for a growing global population. Due to a rise in affluence in developing countries, demand for milk and milk products has grown (FAO, 2023). Milk and milk products are a significant source of protein and fat, as well as certain vitamins and minerals. This is important especially in low-income populations with limited access to milk products, as it provides a vital source of energy for growing children (FAO, 2023). A demand for higher-fat milk products has increased in recent years as studies have indicating the nutritional benefits of milk fat, contrary to popular thought. The Dietary Guidelines for Americans have promoted low or non-fat dairy foods since the first publication in 1980 and have continued to promote this message until the most recent edition in 2020 (U.S. Department of Agriculture, 2020). These claims are largely supported by observational nutritional studies during 1960's and 1970's that showed direct association between saturated fat consumption and increased risk of cardiovascular disease (JAMA, 1961). However recent meta-analyses and reviews have pointed out weaknesses of this claim since it doesn't consider variability of fatty acids with different physiological effects in saturated fat and the effect of food matrix which fatty acids interact with (Astrup et al., 2019). A systematic review and meta-analysis found no associations between intake of saturated fat and all cause mortality, CVD mortality, CHD mortality, ischemic stroke or type 2 diabetes (De Souza et al., 2015). In response to research challenging long standing assumptions on dietary fat and the increased demand for food, dairy farmers have selected for breeds and adjusted feeds to meet demand, reflected by increases in percent milk fat and percent skim solids over the past decade (Terán and Cessna, 2021). In addition to milk fat, demand for

milk protein is projected to grow, as the global milk protein market is projected to almost double from 2021- 2030 (Verified Market Research, 2023). Key markets driving this demand include sports nutrition, infant formula and dairy products. Casein and whey protein supplements have been interest in athletic sports performance and recovery, as high proportions of branched chain amino acids have been shown to increase muscle protein synthesis (Volek et al., 2013). Infant formula companies utilize whey protein to develop ideal protein ratios based on human milk for growing infants. A growing population paired with increasing demands in both milk fat and protein has put pressure on the dairy industry to improve milk production. Since dairy production systems are resource intensive, current approaches need to be optimized to meet this demand.

Nitrogen Efficiency

Supplying feed can contribute up to 60% of total dairy production costs (Connor, 2015). Coinciding with rising costs, improving feed efficiency is a main concern for milk producers. One portion of feedstuff that has ample room for improvement is utilization of dietary nitrogen. Ruminant conversion of dietary nitrogen to production purposes is low, only an estimated 25% of dietary nitrogen is incorporated into milk and the rest being lost in metabolic waste (Arriola Apelo et al., 2014). Dietary protein is a costly ingredient, representing 42% of the cost of lactating cow rations (St-Pierre, 2012). With low conversion rates and high costs, improving nitrogen utilization is a major goal for dairy production systems.

In addition to economic factors, there has been a growing concern about the impacts of excess nitrogen pollution on environmental and human health. Adverse effects of excess nitrogen in the environment include but not limited to, increased nitrate levels in drinking water beyond health standards, eutrophication of water systems and loss of biodiversity. Agriculture-producing states are especially at risk, as a study conducted in central Wisconsin found nitrate

concentrations in groundwater have significantly increased over time (Saad, 2008). Drinking water with increased nitrate levels has been linked to methemoglobinemia, colorectal cancer, thyroid disease and neural tube defects (Ward et al. 2018). To address this issue, nitrogen metabolism in dairy cows has been studied and re-modeled over the years with the goal to improve the efficiency of dairy cows. The National Research Council's publication *Nutrient Requirements of Dairy Cattle* is a report assembled by dairy science experts with the purpose of improving the accuracy of predicting animal performance from nutrient inputs and other variables that affect requirements (NASEM, 2021). This report has a major influence and shapes practices used across the dairy industry. As the report provides evidence-based recommendations, progression of dairy nutrition research is critical to tackle the issue of nitrogen efficiency. The goal of the research described later in this paper is to improve nutritional knowledge and recommendations provided by the NRC regarding nitrogen utilization.

Nitrogen Digestion and Utilization

Predicting nitrogen supply to the mammary gland in dairy cows is a challenging task as additional physiological factors manipulate digestion and flux to peripheral tissues compared to non-ruminants. The presence of microbes in the rumen, reticulum and omasum enables the dairy cow to partially digest indigestible forages and reuse it for microbial growth. The harsh environment of the rumen rapidly degrades protein and is based on the proteolytic activity of the ruminal microflora and type of protein present (Bach, 2005). Microbial crude protein (MCP), protein synthesized by microbes, accounts for most of the total absorbable protein available to the small intestine, thus playing a major role to consider in predicting net nitrogen flux (Lapierre et al., 2006). Volatile fatty acids (VFAs) are the major product of fermentation in the rumen and serve as an energy source for production purposes. Almost all dietary carbohydrates are

fermented to VFAs in the rumen, thus gluconeogenesis provides up to 90% of glucose requirements (Young, 1977). The type and amount of energy source available in the diet for microbial fermentation will thus influence nutrient flux into the mammary gland. After passing the rumen, the primary absorptive site of dietary and microbial nitrogen is the gastrointestinal tract lumen, where the gastrointestinal epithelium actively regulates nutrient metabolism and absorption. Once absorbed, tissues of the portal-drained viscera (PDV) and liver use a significant amount of energy and amino acids, controlling the rate and proportions of metabolites available for peripheral tissues. All these factors converge to complicate the prediction of nitrogen sources available to the mammary gland.

Once passed the liver, amino acids are available for tissue utilization. If amino acids are not incorporated into body proteins or milk proteins, they will be recycled to the liver, where carbon skeletons can be oxidized or used for anabolic processes like gluconeogenesis, and the ammonia is converted to urea and excreted in urine. It is estimated that 50% of nitrogen excreted by lactating dairy cows is due to post-absorption losses (Arriola Apelo et al., 2014). A strategy to reduce post-absorption losses would be to increase the rate of amino acid uptake by the mammary gland, which will reduce recycling of amino acids to the liver. The mammary gland utilizes amino acids for milk protein synthesis, tissue protein accretion and catabolism, with milk protein yield as the primary factor regulating uptake of amino acids (Cant et al., 2018). The rate at which amino acid uptake occurs is dependent mammary blood flow (MBF) and mammary affinity for individual AA. These factors not considered constant when predicting nutrient uptakes from blood. Upregulating the rate of blood flow, amino acid utilization and translation of milk protein are points of regulation that dairy scientists are investigating, as it will lead to increased sequestration of nitrogen in milk. These factors described briefly play a role in

influencing nitrogen metabolism and are integrated into mathematical models to predict nutrient and amino acid availability to the mammary gland.

mTORC1 Signaling

Understanding nutritional regulation of signaling pathways responsible for protein synthesis of the mammary gland is crucial to improve milk protein output and efficiency. The main signaling pathways that converge to regulate milk protein yield include, mTORC1, ISR and GSK3 (Cant et al., 2018). Considered as the main point of regulation for milk protein synthesis, the mTORC1 pathway has been studied the most in dairy cows and will be described briefly. The target of rapamycin (TOR) was first identified by a genetic screen of budding yeast that were resistant to the immunosuppressant rapamycin, followed by identification in humans and other species, suggesting early evolutionary conservation (Tatebe and Shiozaki, 2017). The highly conserved protein complex mTORC1 is a key integration center of cellular nutrient and stress status, promoting anabolic pathways such as ribosomal biogenesis, protein translation and inhibiting autophagy when nutrient conditions are met. Briefly, amino acids inhibit protein GATOR1, activating Rag GTPases which recruit mTORC1 and Rheb to the lysosome (Kennedy and Lamming, 2016). In addition to amino acids, mTORC1 is sensitive to glucose since insulin is required to localize the complex to the lysosome. Insulin removes TSC from the lysosome, an inhibitor of Rheb, which then allows mTORC1 to interact with GTP-bound Rheb and become active (Kennedy and Lamming, 2016). The lysosome serves as the physical site of coordination between amino acid recycling and mTORC1 activation. It has been shown that mTORC1 is stimulated by branched-chain amino acids, but the mechanisms of regulation by specific amino acids are currently under investigation.

TOR signaling within the scope of biomedical research has increased exponentially after it has shown to play a prominent role in human diseases, such as cancer and cardiovascular disease. Although TOR is highly conserved, application of findings from human research to dairy cows isn't straightforward since physiology between non-ruminants and ruminants differs substantially. Thus, basic research on TOR signaling in dairy cows particularly of the mammary gland is needed. Several in vitro experiments have begun to unravel amino acid, energy and endocrine regulation of mammary cell lines and tissue. EAA was shown to increase phosphorylation of mTOR and 4EBP, intracellular EAA concentration and casein synthesis rates in mammary tissue slices independently of energy substrate (Appuhamy et al., 2014). Additionally, Ile and Leu have been shown to independently stimulate mTOR signaling in MAC-T cells and bovine mammary tissue slices (Appuhamy et al., 2012). In addition to amino acids, mTOR is regulated by cellular energy status through the AMPK pathway (Gwinn, 2008). AMPK is activated when ATP levels are low, shifting cellular signaling towards catabolism. Mammary epithelial cells had decreased protein synthesis rates and inhibition of mTORC1 signaling when AMPK was activated (Burgos et al., 2013), demonstrating energy status as another route of mTOR regulation. However, when comparing effects of EAA and AMPK on mTORC1 regulation of protein synthesis rates, EAA activation of mTOR-regulation on protein synthesis was more significant (Appuhamy et.al., 2014).

Many endocrine factors regulate the development and function of the mammary gland. Insulin is regarded to as the most potent anabolic hormone, essential for regulating blood glucose homeostasis. Insulin is intricately connected with mTOR signaling through the P13K/Akt signaling pathway (Kennedy and Lamming, 2016). In a MAC-T cell line, insulin was required for EAA activation of mTORC1 (Pszczolkowski et al., 2020), aligning with previous work in

human skeletal muscle (Drummond et al., 2008). Insulin has also been shown to upregulate mammary AA uptake by increasing mammary blood flow and mammary AA affinity (Pszczolkowski and Arriola Apelo, 2020). Prolactin, with adequate nutrient availability, upregulated mammary protein synthesis via mTOR signaling (Burgos, 2010). Thus, endocrine signaling is yet another input responsible in regulating mTORC1-mediated protein synthesis.

The mTORC1 pathway is under tight regulation of several converging inputs of cellular status. Continuation of molecular work determining the effects of energy, endocrine and amino acid regulation on milk protein synthesis is necessary to develop in vivo strategies stimulating milk protein production.

AA Supplementation

Historically, nutrient requirement systems for lactating dairy cows have predicted milk protein yield based on the limiting AA theory. This proposes the amount of substrate determines the rate of milk protein synthesis, as one limiting factor could stop protein synthesis. Based on this assumption amino acid requirements are often over-predicted and lead to excess nitrogen excretion (Arriola Apelo et al., 2014). Recently, research has sought to determine the ideal balance of dietary amino acids for milk protein synthesis to reduce usage of high-cost dietary protein and improve efficiency. Certain amino acids have been shown to upregulate mTORC1 signaling, thus studies have focused on the effects of these AA on milk protein synthesis in vivo. Jugular infusion of methionine + lysine had positive effects on protein yield and content in high-producing dairy cows (Appuhamy et al., 2011). Likewise, goats infused with glucose plus a mix of isoleucine, leucine, methionine and threonine tended to increase milk protein production, however it was not explained by mTORC1 signaling (Xu et al., 2019). Supplementation of rumen protected methionine in a low protein diet increased milk protein and a mix of rumen

protected amino acids (methionine, leucine, isoleucine and threonine) paired with low protein tended to increased milk protein (Zhao et al., 2019). Based on increasing evidence on the positive effects of balancing for AA, nutrient requirement models have shifted to incorporate independent responses to at least 5 individual AA: Met, Lys, His, Ile, Leu (NASEM, 2021).

Energy Source

There has been less attention on the role of dietary energy source on mammary gland utilization of amino acids in nutrition models. The input of energy in these models is described as a general source of calories (excluding protein) and doesn't consider its role in regulating mammary gland metabolism. Emerging evidence has shown the type of energy profile provided with supplemented amino acids may influence mammary gland utilization of amino acids. Investigating the role of energy source commonly utilizes infusions to bypass the rumen, as it ferments substrates and provides these products as energy to the cow, acting as a confounding factor. Infusion of glucose compared to other types of energy was shown to stimulate milk protein synthesis (Danes et al., 2020). Additionally, infusion of glucose paired with essential AA tended to increase milk protein synthesis (Nichols et al., 2019). Complementing infusion studies, diets rich in starch improved the conversion efficiency of plasma EAA into milk protein compared to fiber diets (Cantalapiedra-Hijar et al., 2015). These findings suggest that glucogenic energy may stimulate mTORC1 mediated protein synthesis in the mammary gland. This is in line with previous in vitro work which demonstrated that insulin potentiates EAA's effect on mTORC1 signaling (Pszczolkowski et al., 2020). Infusion studies draw important conclusions, but they aren't applicable to a large production setting. Comprehensive dietary studies utilizing non-invasive approaches are the next logical approach. Therefore, we designed an experiment to compare response to dietary AA supplementation, balanced for the 5 EAA with independent

effects on milk protein, paired with either a glucogenic or ketogenic energy diet. Our hypothesis was that glucogenic energy would potentiate AA stimulation of milk protein synthesis, reflected by increases of mammary gland extraction and uptake of amino acids. To analyze amino acid utilization, quantitative analysis of plasma amino acids via liquid chromatography mass spectrometry will provide us with precise concentrations of individual amino acids per cow, treatment and timepoint. Internal standards and non-metabolizable markers allow us to determine amino acid concentration, extraction and uptake per treatment to draw conclusions.

Materials and Methods

Treatments. 36 dairy cows were subjected to 4 treatments: Keto-CTRL, Keto+BAA, Gluco-CTRL and Gluco+AA in 4 consecutive periods based on a Latin square design. For the factor of AA supplementation, the CTRL diet was formulated as 10% metabolizable protein (MP) deficient, while the BAA diet met MP, His, Ile, Leu, Lys and Met requirements. Concerning the energy factor, both ketogenic and glucogenic diets had fixed ratios of corn and alfalfa silage, but different concentrate profiles that altered relative starch and fat levels. Each 28-day period consisted of 21 days for diet adaptation and the last week for sample collection. For plasma amino acid analysis, a subset of 20 cows were blood sampled from a coccygeal vessel, representing arterial blood, and from the subcutaneous abdominal vein, representing mammary vein blood, for 6 timepoints staggered between day 25-26 of each period to represent every 90 min sampling. Sampling timepoints were 0700, 1000, and 1300 on day 25, and 0830, 1130, and 1430 h on day 26. Blood samples were collected into heparin tubes (BD vacutainer) and placed on ice immediately. Blood was centrifuged at 4 °C at 2,500 x g for 15 minutes to isolate plasma. Three aliquots per sample were stored in microcentrifuge tubes and stored in -80°C until later analysis.

Amino acid analysis. Plasma (12.5 µg) was combined with ^{13}C universally labeled amino acid mix (2.0 µg; Cambridge Isotope Laboratories, Inc.; catalog no. CLM-1548-PK, CLM-8699-H-PK, CLM-4290-H-PK, CLM-1822-H-PK) as internal standard, followed by deproteinization with 1mol/L perchloric acid (12.5 µg). Amino acids were then isolated through an ion exchange column and derivatized using the EZ:faast kit (Phenomenex, catalog no. KH0–7337), and analyzed by liquid chromatography, electrospray ionization, single quadrupole, mass spectrometry (LCMS-2020, Shimadzu, Kyoto, Japan). Mobile phases were 10 mmol/L ammonium formate in water (A) and 10 mmol/L ammonium formate in methanol (B), with a 68% B gradient for 0-13 min, 83% B gradient for 13-13.01 min and 68% B gradient for 13.02-17 min. Amino acid standard curves were run after at most three batch runs (40-70 samples each batch) to account for potential variability of analysis conditions over time. Individual peaks were checked for correct integrations, ensuring consistency and accuracy throughout runs. Methionine showed inconsistences in chromatograms and was removed from the data set. It will be analyzed with a revised method in the near future.

Calculations. Mammary fractional efficiency of individual amino acids was calculated as (A-V) / A, where A represents arterial plasma and V represents venous plasma. The Fick principle was utilized to determine mammary plasma flow (MPF), using Phe+Tyr as non-metabolizable markers (Cant and McBride, 1995). Concentrations of Phe and Tyr in de novo synthesized milk proteins were estimated to be 51.9 and 58.7 g/kg respectively (Larsen et al., 2015).

Statistical analysis. AA were analyzed in individual samples and averaged per cow, vessel and period. Data management and calculations for AA concentration, blood flow, mammary fractional extraction (%) and mammary uptake was performed in RStudio (2022.07.2).

Final AA data was analyzed with PROC MIXED in SAS 9.4 (SAS Institute, Inc.). The model contained fixed effects of energy source, balanced AA supplementation, period, square and random effect of cow. If observations were greater than three standard deviations away from the mean, they were removed from the data set as outliers.

Results

Table 1 represents circulating plasma concentrations of individual AA. Cows consuming a KE diet presented higher levels of EAA except Thr and Phe. Among NEAA, Ala levels were high while Gln and Ser low. No other NEAA had significant effects with energy source. Considering AA level, BAA treatment increased levels of most EAA. For NEAA, Gln, Pro and Tyr increased while Ala, Glu, Gly and Ser decreased. There was a significant interaction of Leu increasing more in response to BAA under a KE diet compared to GE. There were no other significant interactions seen between AA and energy source.

Table 1. Circulating plasma concentration of AA in lactating dairy cows in response to energy source and metabolizable AA level

	Energy source			AA level			Interactions				
							GLUCO		Keto		
Item	**GLUC**	**KET**	***P***	**CTL**	**AA**	***P***	**CTL**	**AA**	**CTL**	**AA**	***P***
EAA											
Arg	60	76	<0.001	67	69	0.50	59	61	75	76	0.91
His	23	27	0.01	19	31	<0.001	18	29	21	33	0.58
Ile	89	112	<0.001	100	100	0.92	89	88	110	113	0.63
Leu	153	167	<0.01	117	203	<0.001	114	192	120	214	0.04
Lys	82	97	<0.001	83	96	<0.001	78	87	88	105	0.10
Phe	44	45	0.67	40	48	<0.001	41	47	40	49	0.15
Thr	95	99	0.14	105	89	<0.001	104	86	105	92	0.24
Trp	41	45	0.01	46	40	<0.001	44	38	48	42	0.90
Val	173	208	<0.001	178	202	<0.001	161	184	195	220	0.82
NEAA											
Ala	295	313	0.05	316	292	0.01	306	285	327	300	0.71
Asn	57	58	0.34	57	58	0.76	57	56	57	59	0.21
Asp	6	6	0.38	6	6	0.57	6	6	6	6	0.19

Glu	58	55	0.08	59	54	<0.001	61	54	57	54	0.17
Gln	225	198	<0.001	206	217	0.04	225	225	187	209	0.05
Gly	401	388	0.29	448	341	<0.001	465	337	432	344	0.11
Pro	86	87	0.72	77	95	<0.001	77	95	77	96	0.80
Ser	94	86	0.01	97	83	<0.001	104	84	91	82	0.06
Tyr	79	81	0.25	75	85	<0.001	75	83	75	86	0.24

Table 2 shows mammary fractional extraction of individual AA. Under the KE diet considering EAA, cows had increased extraction of Leu and Phe and decreased extraction of Ile and Lys. For NEAA, there was increased extraction of Gln and Ser, with tendency for Glu. Under BAA supplementation considering EAA, there was increased extraction of Arg, Ile, Thr and Trp and tendency for Val and decreased extraction of His and Leu. For NEAA, there was increased extraction of Ala, Asn, Gln, and Ser, with tendency for Pro. The only significant interaction was seen for Ile; its mammary fractional extraction increased more in response to BAA under a KE diet compared to the GE diet. There were no other significant interactions seen between AA and energy source.

Table 2. Mammary fractional extraction (%) of AA in lactating dairy cows in response to energy source and AA level.

							Interactions				
	Energy source			AA level			GLUCO		KETO		
Item	GLUCO	KETO	*P*	CTL	AA	*P*	CTL	AA	CTL	AA	*P*
EAA											
Arg	51.6	49.2	0.12	48.3	52.5	0.01	49.3	53.8	47.2	51.2	0.87
His	50.1	48.4	0.55	54.0	44.5	0.002	55.7	44.6	52.4	44.4	0.59
Ile	38.1	32.0	0.01	30.5	39.6	<0.001	35.7	40.6	25.3	38.7	0.05
Leu	35.7	40.6	0.03	45.7	34.5	<0.001	47.1	36.8	44.3	32.1	0.58
Lys	66.5	63.3	0.02	64.1	65.7	0.24	65.7	67.4	62.6	64.0	0.90
Phe	47.0	49.6	0.05	47.9	48.7	0.55	46.6	47.5	49.2	50.0	0.97
Thr	27.7	28.3	0.67	23.4	32.6	<0.001	23.2	32.3	23.7	32.9	0.97
Trp	12.8	11.3	0.25	10.0	14.1	<0.01	11.3	14.3	8.7	13.9	0.41
Val	26.2	23.4	0.06	23.6	25.9	0.11	25.2	27.1	22.0	24.8	0.75
NEAA											
Ala	13.4	11.0	0.13	10.5	14.0	0.03	11.1	15.7	9.9	12.2	0.47
Asn	24.4	25.6	0.45	22.3	27.6	<0.01	21.8	27.0	22.8	21.8	0.93
Asp	44.4	40.2	0.25	42.1	42.5	0.91	45.4	43.4	38.8	41.6	0.49
Glu	62.1	64.4	0.07	63.1	63.3	0.85	62.1	62.0	64.0	64.7	0.74
Gln	25.4	28.6	0.01	25.7	28.3	0.04	24.1	26.8	27.4	29.9	0.92
Gly	1.7	0.8	0.72	0.4	2.1	0.50	2.0	1.5	-1.1	2.8	0.40
Pro	16.6	17.1	0.69	15.9	17.8	0.14	15.6	17.6	16.2	18.0	0.96

Ser	22.3	29.5	<0.001	19.9	31.9	<0.001	16.7	27.9	23.0	35.9	0.65
Tyr	34.4	35.9	0.32	34.2	36.2	0.19	33.7	35.1	34.6	37.3	0.66

Table 3 shows mammary uptake of individual AA (blood flow considered). Under the KE diet considering EAA, there was increased uptake of Arg and decreased uptake of Leu, with tendency for Trp. For NEAA, there was increased uptake of Ser and decreased uptake of Ala and Asp, with tendency for Gln. Under BAA supplementation considering EAA, there was increased uptake of His, Ile, Leu, Lys, Phe, Thr and Val, with tendency for Trp. For NEAA, there was increased uptake of Asn, Gln, Pro, Ser and Tyr and decreased uptake of Glu. The only interaction between energy source and AA on mammary uptake was seen with Ile; its mammary uptake increased more in response to BAA under a KE diet compared to GE.

Table 3. Mammary uptake of AA in lactating dairy cows in response to energy source and AA level.

	Energy source			AA level			Interactions				
							GLUCO		KETO		
Item	GLUCO	KETO	*P*	CTL	AA	*P*	CTL	AA	CTL	AA	*P*
EAA											
Arg	24.1	26.9	0.01	24.9	26.1	0.30	23.4	24.7	26.4	27.5	0.30
His	8.2	8.3	0.71	7.6	8.9	<0.001	7.7	8.7	7.5	9.1	0.38
Ile	25.3	23.5	0.18	21.6	27.2	<0.001	24.2	26.5	19.1	27.8	0.02
Leu	48.7	43.9	0.02	41.8	50.7	<0.001	43.2	54.2	40.4	47.3	0.30
Lys	42.6	44.4	0.18	41.4	45.6	0.002	41.0	44.2	41.7	47.0	0.41
Phe	15.8	15.7	0.81	14.7	16.9	<0.001	14.9	16.7	14.5	17.0	0.34
Thr	19.5	19.5	0.98	18.1	20.8	0.002	18.1	20.9	18.1	20.8	0.96
Trp	4.1	3.4	0.09	3.5	4.1	0.13	3.9	4.3	3.0	3.9	0.50
Val	34.5	34.6	0.96	31.7	37.4	<0.001	31.3	37.8	32.2	37.0	0.57
NEAA											
Ala	30.3	22.7	0.04	24.3	28.7	0.23	27.3	33.2	21.3	24.2	0.67
Asn	10.4	10.3	0.96	9.4	11.3	0.002	9.4	11.4	9.4	11.3	0.95
Asp	2.0	1.7	0.05	1.9	1.8	0.49	2.1	1.9	1.7	1.7	0.53
Glu	27.5	26.0	0.20	28.6	25.0	0.004	29.4	25.7	27.7	24.3	0.92
Gln	44.1	41.2	0.09	41.0	44.3	0.05	42.8	45.4	39.1	43.2	0.66
Gly	8.6	0.04	0.26	4.1	4.5	0.96	13.3	3.9	-5.1	5.2	0.19
Pro	10.6	10.4	0.79	9.1	11.9	<0.001	9.0	12.2	9.3	11.5	0.42
Ser	14.1	16.8	0.02	12.6	18.3	<0.001	10.9	17.3	14.2	19.3	0.53

Tyr	20.7	20.6	0.73	19.4	21.9	<0.001	19.6	21.8	19.2	21.9	0.56

Discussion

The goal of this study was to investigate the role of energy source on mammary gland protein synthesis. A dietary approach designed to overcome confounding factors from rumen fermentation was used to determine its efficacy in a production setting. Milk protein response to energy source was contrary to our prediction since we observed higher levels of milk protein g/d with ketogenic compared to glucogenic energy. Previous findings in literature are inconsistent on the effect of fat-based diets on milk protein yield. Some have shown increases in milk protein (Nichols et al., 2019) while others have seen the opposite (Danes et al., 2020; Cantalapiedra-Hijar et al., 2015). This is most likely due to differences in diet formulation and method of treatment delivery (dietary or infusion). Considering the effect of AA level, balancing for amino acids significantly increased milk protein g/d and protein %, which is commonly reported in literature. Energy source and AA level independently affected milk protein g/d, but there was no interaction between the two treatment factors.

Circulating plasma AA concentrations were significantly different between energy source, as the ketogenic diet had increases in most EAA and decreases in some NEAA. Among EAA, ketogenic diets significantly increased concentration of Lys, Arg, Ile, Leu and Val, and tended for His and Trp. The only increase of NEAA with ketogenic energy was Ala. In contrast, concentrations of Gln and Ser significantly decreased while Gly and Glu tended to decrease with ketogenic energy. Increased concentration of group 2 AA (Arg, Ile, Leu, Lys and Val) paired with decreases of Gln and Ser have been reported with increased dietary fat (Nichols et al. 2019). Thus, energy source may play a role in regulating the utilization of group 2 AA for mammary gland metabolism. Considering the effect of energy source, a general trend was observed when

plasma amino acid concentrations increased, mammary fractional extraction decreased in response for Arg, Ile, Lys, Trp, Val and Ala. When concentrations decreased for Gln, Ser and Phe, mammary fractional extraction increased in response. Interestingly, Leu did not follow this trend, its concentration and mammary extraction simultaneously increased with ketogenic energy. These results demonstrate the ability of the mammary gland to tightly regulate AA balance in response to changing supply in plasma.

Energy source had few significant effects on mammary AA uptake. There was an increased uptake of serine and arginine, while tending to increase for Lys with ketogenic energy. While uptake of Ala decreased and Asp, Gln and Trp tended to decrease. Reduced uptake in response to higher plasma concentrations has been reported and reflects the ability of the mammary gland to maintain AA balance. Although there is not much documented on the role of energy source on AA uptake, an increase in serine uptake in response to fat has been previously documented (Nichols et al. 2019). Increased uptake of arginine may be related to increased MUN observed with ketogenic energy. In porcine mammary tissue, excess uptake of arginine served as a substrate for milk urea synthesis (O'Quinn et al., 2002). Of the amino acids that increased in uptake, they may have not been directed towards milk protein synthesis.

Although circulating concentrations of amino acids increased with ketogenic energy in general, lack of improved mammary uptake failed to improve nitrogen efficiency. Significant increases in PUN mg/dL and urinary N g/d suggest increased recycling of excess amino acids via the liver. Further molecular work such as western blotting or RNA sequencing on mammary, liver and adipose tissue will determine the activity of mammary gland transporters and enzymes related to amino acid metabolism. This will provide a deeper understanding of amino acid dynamics presented in this paper. Another route of investigation would be to determine the role

of energy source on microbial composition and crude protein output since the rumen dictates nutrient availability to the mammary gland.

Conclusion

With the given parameters of this experiment, ketogenic energy increased concentrations of most EAA and increased group 2 AA mammary metabolism. Lack of significant effects of energy on mammary gland extraction and uptake reveal that energy source does not play a significant role in regulating sequestration of amino acids. Results from this study demonstrate the difficultly of translating findings from infusion studies to dietary-based approaches. These findings will contribute to greater knowledge of the role of energy source on mammary gland amino acid metabolism and create efficient dietary recommendations used in the dairy industry.

References

Apelo, S. I. A., Singer, L. M., Ray, W. K., Helm, R. F., Lin, X. Y., McGilliard, M. L., St-Pierre, N. R., & Hanigan, M. D. (2014). Casein synthesis is independently and additively related to individual essential amino acid supply. *Journal of Dairy Science*, *97*(5), 2998–3005.

Appuhamy, J. A., Nayananjalie, W. A., England, E. M., Gerrard, D. E., Akers, R. M., & Hanigan, M. D. (2014). Effects of AMP-activated protein kinase (AMPK) signaling and essential amino acids on mammalian target of rapamycin (mTOR) signaling and protein synthesis rates in mammary cells. *Journal of Dairy Science*, *97*(1), 419–429.

Appuhamy, J. A., Knapp, J. R., Becvar, O., Escobar, J., & Hanigan, M. D. (2011). Effects of jugular-infused lysine, methionine, and branched-chain amino acids on milk protein synthesis in high-producing dairy cows. *J. Dairy Sci.*, *94*, 1952–1960.

Appuhamy, J. A., Knoebel, N. A., Nayananjalie, W. A., Escobar, J., & Hanigan, M. D. (2012). Isoleucine and leucine independently regulate mTOR signaling and protein synthesis in MAC-T cells and bovine mammary tissue slices. *J. Nutr.*, *142*, 484–491.

Bach, A., Calsamiglia, S., & Stern, M. D. (2005). Nitrogen Metabolism in the Rumen. *Journal of Dairy Science*, *88*(S), E9–E21.

Burgos, S. A., Dai, M., & Cant, J. P. (2010). Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway. *J. Dairy Sci.*, *93*, 153–161.

Burgos, S. A., Kim, J. J. M., Dai, M., & Cant, J. P. (2013). Energy depletion of bovine mammary epithelial cells activates AMPK and suppresses protein synthesis through inhibition of mTORC1 signaling. *Hormone and Metabolic Research*, *45*(3), 183–189.

Cant, J. P., Kim, J. J. M., Cieslar, S. R. L., & Doelman, J. (2018). Symposium review: Amino acid uptake by the mammary glands: Where does the control lie?1. *Journal of Dairy Science*, *101*(6), 5655–5666.

Cantalapiedra-Hijar, G., Ortigues-Marty, I., & Lemosquet, S. (2015). Diets rich in starch improve the efficiency of amino acids use by the mammary gland in lactating Jersey cows. *Journal of Dairy Science*, *98*(10), 6939–6953.

Central Committee for Medical and Community Program of the American Heart Association. (1961). Dietary Fat and Its Relation to Heart Attacks and Strokes. *JAMA*, *175*(5), 389.

Connor, E. E. (2015). Invited review: Improving feed efficiency in dairy production: challenges and possibilities. *Animal*, *9*(3), 395–408.

Danes, M. A. C., Hanigan, M. D., Arriola Apelo, S. I., Dias, J. D. L., Wattiaux, M. A., & Broderick, G. A. (2020). Post-ruminal supplies of glucose and casein, but not acetate, stimulate milk protein synthesis in dairy cows through differential effects on mammary metabolism. *Journal of Dairy Science*, *103*(7), 6218–6232.

De Souza, R. J., Mente, A., Maroleanu, A., Cozma, A. I., Ha, V., Kishibe, T., Uleryk, E., Budylowski, P., Schünemann, H., Beyene, J., & Anand, S. S. (2015). Intake of saturated and trans unsaturated fatty acids and risk of all cause mortality, cardiovascular disease, and type 2 diabetes: Systematic review and meta-analysis of observational studies. *BMJ*, h3978.

Dijk, M. van, Morley, T., Rau, M. L., & Saghai, Y. (2021). A meta-analysis of projected global food demand and population at risk of hunger for the period 2010–2050. *Nature Food 2021 2:7*, *2*(7), 494–501.

Drummond, M. J., Dreyer, H. C., Pennings, B., Fry, C. S., Dhanani, S., Dillon, E. L., Sheffield-Moore, M., Volpi, E., & Rasmussen, B. B. (2008). Skeletal muscle protein anabolic

response to resistance exercise and essential amino acids is delayed with aging. *Journal of Applied Physiology*, *104*(5), 1452–1461.

Food and Agriculture Organization of the United Nations. (n.d.). Gateway to dairy production and products. *Milk and Milk Products*.

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., Turk, B. E., & Shaw, R. J. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Molecular Cell*, *30*(2), 214–226.

Kennedy, B. K., & Lamming, D. W. (2016). The Mechanistic Target of Rapamycin: The Grand ConducTOR of Metabolism and Aging. *Cell Metabolism*, *23*(6), 990–1003.

Lapierre, H., Pacheco, D., Berthiaume, R., Ouellet, D. R., Schwab, C. G., Dubreuil, P., Holtrop, G., & Lobley, G. E. (2006). What is the True Supply of Amino Acids for a Dairy Cow? *Journal of Dairy Science*, *89*, E1–E14.

National Academies of Sciences, Engineering, and Medicine. (2021). *Nutrient Requirements of Dairy Cattle: Eighth Revised Edition*. Washington, DC: The National Academies Press.

Nichols, K., Bannink, A., Doelman, J., & Dijkstra, J. (2019). Mammary gland metabolite utilization in response to exogenous glucose or long-chain fatty acids at low and high metabolizable protein levels. *Journal of Dairy Science*, *102*(8), 7150–7167.

O'Quinn, P. R., Knabe, D. A., & Wu, G. (2002). Arginine catabolism in lactating porcine mammary tissue2. *Journal of Animal Science*, *80*(2), 467–474.

Pszczolkowski, V. L., & Arriola Apelo, S. I. (2020). The market for amino acids: Understanding supply and demand of substrate for more efficient milk protein synthesis. *Journal of Animal Science and Biotechnology*, *11*(1), 108.

Pszczolkowski, V. L., Zhang, J., Pignato, K. A., Meyer, E. J., Kurth, M. M., Lin, A., & Arriola Apelo, S. I. (2020). Insulin potentiates essential amino acids effects on mechanistic target of rapamycin complex 1 signaling in MAC-T cells. *Journal of Dairy Science*, *103*(12), 11988–12002.

Saad, D. A. (2008). Agriculture-Related Trends in Groundwater Quality of the Glacial Deposits Aquifer, Central Wisconsin. *Journal of Environmental Quality*, *37*(S5), S-209.

St-Pierre, N. R. (2012). The costs of nutrients, comparison of feedstuffs prices and the current dairy situation. The Ohio State University Extension Buckeye News.

Tatebe, H., & Shiozaki, K. (2017). Evolutionary Conservation of the Components in the TOR Signaling Pathways. *Biomolecules*, *7*(4), 77.

Terán, A., & Cessna, J. (2021, August 9). *Farm Milk Components and Their Use Among Dairy Products Have Shifted Over Time*.

U.S. Department of Agriculture and U.S. Department of Health and Human Services. *Dietary Guidelines for Americans, 2020-2025*. 9th Edition. December 2020.

Verified Market Research. (n.d.). *Global Milk Protein Market Size By Type (Milk Protein Concentrates, Milk Protein Isolates), By Livestock (Cow, Buffalo), By Application (Sports Nutrition, Infant Formula, Dairy Products), By Geographic Scope And Forecast* (No. 22573; p. 202).

Volek, J. S., Volk, B. M., Gómez, A. L., Kunces, L. J., Kupchak, B. R., Freidenreich, D. J., Aristizabal, J. C., Saenz, C., Dunn-Lewis, C., Ballard, K. D., Quann, E. E., Kawiecki, D. L., Flanagan, S. D., Comstock, B. A., Fragala, M. S., Earp, J. E., Fernandez, M. L., Bruno, R. S., Ptolemy, A. S., … Kraemer, W. J. (2013). Whey Protein Supplementation During Resistance Training Augments Lean Body Mass. *Journal of the American College of Nutrition*, *32*(2), 122–135.

Ward, M., Jones, R., Brender, J., De Kok, T., Weyer, P., Nolan, B., Villanueva, C., & Van Breda, S. (2018). Drinking Water Nitrate and Human Health: An Updated Review. *International Journal of Environmental Research and Public Health*, *15*(7), 1557.

Xu, L. B., Hanigan, M. D., Lin, X. Y., Li, M. M., Yan, Z. G., Hu, Z. Y., Hou, Q. L., Wang, Y., Shi, K. R., & Wang, Z. H. (2019). Effects of jugular infusions of isoleucine, leucine, methionine, threonine, and other amino acids on insulin and glucagon concentrations, mammalian target of rapamycin (mTOR) signaling, and lactational performance in goats. *Journal of Dairy Science*, *102*(10), 9017–9027.

Young, J. W. (1977). Gluconeogenesis in Cattle: Significance and Methodology. *Journal of Dairy Science*, *60*(1), 1–15.

Zhao, K., Liu, W., Lin, X. Y., Hu, Z. Y., Yan, Z. G., Wang, Y., Shi, K. R., Liu, G. M., & Wang, Z. H. (2019). Effects of rumen-protected methionine and other essential amino acid supplementation on milk and milk component yields in lactating Holstein cows. *Journal of Dairy Science*, *102*(9), 7936–7947.