Increasing the availability of threonine, isoleucine, valine, and leucine relative to lysine while maintaining an ideal ratio of lysine:methionine alters mammary cellular metabolites, mammalian target of rapamycin signaling, and gene transcription

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ABSTRACT

Amino acids not only serve as precursors for protein synthesis but also function as signaling molecules that can regulate the mammalian target of rapamycin (mTOR) pathway. Methionine and Lys are the most-limiting AA for milk production and a ratio of ~3:1 Lys:Met in the metabolizable protein has been determined to be ideal. Besides Met and Lys, recent studies have evaluated Ile, Leu, Val, and Thr as potentially limiting for milk protein synthesis. The objective of this experiment was to determine if varying the ratio of Lys:Thr, Lys:Ile, Lys:Val, and Lys:Leu while maintaining an ideal ratio of Lys:Met and fixed ratio of other essential AA (IPAA) elicits changes in intracellular metabolites, gene transcription related to protein synthesis, and phosphorylation status of mTOR pathway proteins. Immortalized bovine mammary epithelial cell line (MAC-T) cells were incubated for 12 h ($n = 5$ replicates/treatment) with IPAA (2.9:1 Lys:Met; 1.8:1 Lys:Thr; 2.38:1 Lys:His; 1.23:1 Lys:Val; 1.45:1 Lys:Ile; 0.85:1 Lys:Leu; 2.08:1 Lys:Arg) or IPAA supplemented with Thr, Ile, Val, and Leu to achieve a Lys:Thr 1.3:1 (LT1.3), Lys:Ile 1.29:1 (LI1.29), Lys:Val 1.12:1 (LV1.12), or Lys:Leu 0.78:1 (LL0.78). Compared with IPAA, metabolomics via gas chromatography-mass spectrometry revealed that increases in availability of Thr, Ile, Val, and Leu led to greater concentrations of essential AA (Leu, Ile, Thr), nonessential AA (Gly, Glu, Gln, Ser, Pro, Asp), and various metabolites including uric acid, phosphoric acid, *N*-acetylglutamic acid, and intermediates of glycolysis and the tricarboxylic acid cycle. Compared with other treatments, LV1.12 led to greater phosphorylation

status of serine/threonine kinase B (Akt), mTORC1, and ribosomal protein S6 and lower phosphorylation of α subunit of eukaryotic translation initiation factor 2. In addition, LV1.12 upregulated abundance of *CSN2* and both the abundance and promoter methylation of *CSN1S1*. Although LI1.29 led to the second highest response in mTORC1 phosphorylation status, it resulted in the lowest phosphorylation of Akt and eEF2 and mRNA abundance of *CSN2* and various AA transporters (*SLC7A5*, *SLC36A1*, *SLC38A2*, *SLC38A9*, *SLC43A1*). Overall, data indicate that an increase in Val at an ideal ratio of Lys:Met could further enhance milk protein synthesis by altering intracellular concentrations of essential AA and metabolites that could play a regulatory role, increasing phosphorylation status of mTORC1 and key signaling proteins, and upregulation of AA transporters.

Key words: essential amino acid ratio, metabolomics, milk protein synthesis, lactation

INTRODUCTION

It has been estimated that only up to 38% of the total AA flux across the mammary gland is used for milk protein synthesis; hence, additional capture of dietary N is a challenge of economic and environmental importance facing the modern dairy industry (Huhtanen and Hristov, 2009; Toerien et al., 2010; Wang et al., 2010). Recent studies demonstrated that balancing EAA profiles could improve milk protein synthesis (Haque et al., 2015). Methionine and Lys are the most-limiting AA for milk protein synthesis, and a ratio of ~3:1 of Lys to Met was determined to be ideal in the context of in vitro casein protein synthesis through alterations in mTOR pathway phosphorylation status and mRNA abundance (Nan et al., 2014). In addition to Lys and Met, recent studies revealed that Ile, Leu, Val, and Thr

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could be limiting for optimal protein synthesis (Appuhamy et al., 2011b, 2012; Doelman et al., 2015; Zhou et al., 2015).

It is now well known that AA not only serve as precursors for milk protein but also function as signaling molecules to regulate the mammalian target of rapamycin (**mTOR**) pathway and, in turn, protein synthesis (Yang et al., 2006; Xie and Proud, 2014). Upon activation by AA, mammalian target of rapamycin complex 1 (**mTORC1**) phosphorylates eukaryotic initiation factor 4E binding protein 1 (**4EBP1**) and ribosomal protein S6 kinase (**S6K1**; Appuhamy et al., 2011a) followed by ribosomal protein S6 (**RPS6**), α subunit of eukaryotic translation initiation factor 2 (**eIF2 α**), and eukaryotic translation elongation factor 2 (**eEF2**) with the end result being the stimulation of mRNA translation initiation and elongation (Doerks et al., 2002; Browne and Proud, 2004; Kimball and Jefferson, 2006; Shimobayashi and Hall, 2014).

The serine/threonine kinase Akt plays an important role in the regulation of mTORC1 activity and forms part of the phosphatidylinositol 3-phosphate kinase (**PI3K**)-Akt-mTOR pathway (Inoki et al., 2002; Xie and Proud, 2014). The most consistent response of mTORC1 to AA is an increase in activity with Leu (Jewell et al., 2013). It is still unclear, however, whether bovine mammary cells respond to increases in the availability of other EAA when the Lys:Met ratio is maintained at the ideal level.

In this study, the general hypothesis was that increasing the in vitro availability of Ile, Leu, Val, and Thr while maintaining an ideal ratio of Lys:Met in mammary cells could influence the phosphorylation status of the mTOR signaling pathway along with gene transcription and intracellular metabolites. Thus, to address this hypothesis we cultured the immortalized bovine mammary epithelial cell line (**MAC-T**), a nonlactating mammary cell line, with various ratios of Lys:Thr, Lys:Ile, Lys:Val, or Lys:Leu and used metabolomics via GC-MS, Western blot, gene transcription, and casein promoter methylation analyses to determine holistic biological responses (Lor et al., 2013).

MATERIALS AND METHODS

Cell Culture

The cell culture was performed using the immortalized MAC-T cell line. The MAC-T cells were incubated in culture dishes (1.8×10^6 cells, 100 mm) to generate enough biological material for metabolomics, and on 6-well plates (0.2×10^6 cells/well) to isolate RNA and protein for Western blot. In both instances, cells were incubated at 37°C in an atmosphere of 5% CO₂ and air,

and grown to approximately 90% confluence before imposing treatments exactly as described by Kadegowda et al. (2009).

Treatments and Experimental Design

Details of treatments and experimental design are included in Supplemental Materials and Table S1 (<https://doi.org/10.3168/jds.2017-13707>). Three independent batches of cell culture were performed to generate material for metabolomics, mRNA abundance, protein abundance, and phosphorylation status. In brief, MAC-T cells were cultured overnight without serum and then treated for 12 h (Li et al., 2017) with 5 treatments ($n = 5$ replicates/treatment) in medium without serum. A lactogenic medium was used for treatment analyses according to a previous publication from one of our laboratories (Kadegowda et al., 2009), except that minimum essential medium/Earle's balanced salts HyQ was replaced by high-glucose Dulbecco's modified Eagle's medium without EAA (custom made by Gibco, Thermo Fisher Scientific, Waltham, MA). Individual EAA (L-isomer, Sigma-Aldrich, St. Louis, MO) were added into the custom high-glucose Dulbecco's modified Eagle's medium.

The control medium contained the ideal profile of Lys:Met and fixed ratios of other EAA (IPAA, Lys:Met 2.9:1; Lys:Thr 1.8:1; Lys:His 2.38:1; Lys:Val 1.23:1; Lys:Ile 1.45:1; Lys:Leu 0.85:1; Lys:Arg 2.08:1) and treatment medium was prepared using IPAA plus different concentrations of Thr, Ile, Val, and Leu to achieve Lys:Thr 1.3:1 (**LT1.3**), Lys:Ile 1.29:1 (**LI1.29**), Lys:Val 1.12:1 (**LV1.12**), and Lys:Leu 0.78:1 (**LL0.78**; Table 1). The IPAA was designed to resemble recommendations from NRC (2001) and Haque et al. (2012, 2013). The lower ratios of Lys to Val, Lys to Ile, and Lys to Leu were to enhance Val, Ile, and Leu supply to a level demonstrated to elicit positive responses in milk protein (Haque et al., 2012, 2015). Similarly, the chosen ratio of Thr to Phe was to maintain the ratio as close as possible to the theoretical optimal (~1.0:1.0) as discussed in Haque et al. (2012). The Lys to Thr ratios were chosen based on data from Prizant and Barash (2008). By changing the supply of these AA while holding Lys constant, we sought to determine individual effects that might help explain in vivo responses in the literature (Haque et al., 2012, 2013, 2015).

RNA Extraction and Real-Time PCR Analysis

The RNA extraction and real-time PCR was performed exactly as described previously (Bionaz and Lor, 2008). In brief, total RNA was obtained using the RNeasy Mini columns (Qiagen, Valencia, CA). The

Table 1. Calculated AA composition of experimental treatments

Item	Treatment ¹				
	IPAA	LT1.3	LI1.29	LV1.12	LL0.78
Lys (μg/mL)	175	175	175	175	175
Met (μg/mL)	60	60	60	60	60
Lys/Met	2.9:1	2.9:1	2.9:1	2.9:1	2.9:1
Thr (μg/mL)	97	125	97	97	97
Phe (μg/mL)	93	119	93	93	93
Thr/Phe	1.05:1	1.05:1	1.05:1	1.05:1	1.05:1
Lys/Thr	1.8:1	1.3:1	1.8:1	1.8:1	1.8:1
His (μg/mL)	74	74	74	74	74
Lys/His	2.38:1	2.38:1	2.38:1	2.38:1	2.38:1
Val (μg/mL)	142	142	142	156	142
Lys/Val	1.23:1	1.23:1	1.23:1	1.12:1	1.23:1
Ile (μg/mL)	121	121	136	121	121
Lys/Ile	1.45:1	1.45:1	1.29:1	1.45:1	1.45:1
Leu (μg/mL)	206	206	206	206	225
Lys/Leu	0.85:1	0.85:1	0.85:1	0.85:1	0.78:1
Arg (μg/mL)	84	84	84	84	84
Lys/Arg	2.08:1	2.08:1	2.08:1	2.08:1	2.08:1
Trp (μg/mL)	16	16	16	16	16
Cys (μg/mL)	63	63	63	63	63
Gln (μg/mL)	584	584	584	584	584
Gly (μg/mL)	30	30	30	30	30
Ser (μg/mL)	42	42	42	42	42
Tyr (μg/mL)	104	104	104	104	104

¹IPAA = ideal profile of Lys:Met and fixed ratio of other EAA; LT1.3 = Lys:Thr ratio of 1.3:1; LI1.29 = Lys:Ile ratio of 1.29:1; LV1.12 = Lys:Val ratio of 1.12:1; LL0.78 = Lys:Leu ratio of 0.78:1. Based on NRC (2001), Haque et al. (2012, 2013).

RNA concentrations and integrity were determined by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples for subsequent analysis had an RNA integrity number > 8.0, and a portion of each was diluted to 100 ng/μL with RNase-free water before cDNA synthesis. One hundred nanograms of RNA was used for cDNA synthesis, and the quantitative PCR was performed as described previously. Primers were designed and verified based on established protocols in the Loor laboratory (Bionaz and Loor, 2011). Three control genes (*GADPH*, *UXT*, and *RPS9*) were used for normalization of target gene abundance. The primer information is reported in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-13707>).

Western Blot

Protein samples from each of the 5 replicates per treatment was used for Western blot analysis as previously described with modifications (Hosseini et al., 2015). Each biological replicate (n = 5/treatment) had 1 technical replicate such that 5 biological replicates were run in the same gel along with the target protein and the internal control (GAPDH). Therefore, the 5 technical replicates were run in 5 different gels. Twenty

micrograms of total protein and 4 × Laemmli sample buffer (#161-0747, Bio-Rad, Hercules, CA) were mixed at a ratio of 3:1 (wt:vol) and denatured at 95°C for 10 min. Proteins were separated using the 10% SDS/PAGE with 120 V for 120 min. Electrophoretic transfer to PVDF membranes (#162-0261, Bio-Rad) was performed in the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (#170-3940, Bio-Rad), and PVDF membranes were incubated with primary antibody overnight at 4°C after blocking for 1 h with 5% skim milk at room temperature. The PVDF membranes were then washed 6 times (5 min/time) and incubated with goat polyclonal secondary antibody for 1 h at room temperature before washing. The visualization of PVDF membranes was performed using the ChemiDoc MP System (Bio-Rad) with Clarity Western ECL Substrate (#170-5060, Bio-Rad) reagent. The Image Lab software (version 3.0) was used for relative quantification of proteins (arbitrary units). All Western data were standardized by the internal reference protein in the same well (e.g., target protein abundance = total target protein intensity/GAPDH intensity). The phosphorylation status was calculated as the ratio of phosphorylated protein/total protein. The antibody list is reported in Supplemental Table S2 (<https://doi.org/10.3168/jds.2017-13707>). The GAPDH was used to normalize target protein abundance.

Promoter Region-Specific DNA Methylation

The DNA extraction and methylation analysis of *MTORC1* and *CSN1S1* were performed as described previously (Osorio et al., 2016). In brief, DNA extraction and cleaning was performed with the DNeasy Blood and Tissue Kit (catalog no. 69504, Qiagen) and Genomic DNA Clean and Concentrator Kit (catalog no. D4013, Zymo Research, Irvine, CA). Identification of gene promoter region specific methylation containing CpG islands was based on the OneStep qMethyl kit (catalog no. D5310, Zymo Research) protocol. Non-methylated DNA (negative control) was obtained by whole-genome amplification, and methylated control (positive control) was obtained by methylase reaction. The primers used in the present study were designed to cover the sections in the promoter region. Primers for the promoter region of *MTORC1* and *CSN1S1* covered from -1,802 to -1,631 bp and from -860 to -618 bp, and contained 2 CpG sites specific for the Methylation Sensitive Restriction Enzyme in the OneStep qMethyl kit. The primer list for methylation is reported in Supplemental Table S3 (<https://doi.org/10.3168/jds.2017-13707>).

GC-MS Analysis

The GC-MS analysis was based largely on a previous report (Levengood et al., 2015). Briefly, the extraction of metabolites was performed by sonicating each replicate in 1 mL of methanol (70%) 5 times (each time for 1 min, 30 s on, then 30 s off) with the QSonica Q700 homogenizer (QSonica LLC, Newton, CT). Before derivatization, 5 μ L of the internal standard (hentriacontanoic acid, 10 mg/mL) was added to each sample supernatant. Metabolomics analysis was performed using a GC-MS system (Agilent Inc.) consisting of an Agilent 7890 gas chromatograph, an Agilent 5975 MSD and a HP 7683B autosampler. All spectra chromatogram peaks were compared with the AMDIS 2.71 (NIST, Gaithersburg, MD) software and a custom-built database. Before data mining, those peaks that could not be matched with the available database were removed. Subsequently, the normalization of all data using the internal standard and cell dry weight was performed. Instrument variability across all samples was within the accepted range of 5%.

Data Analysis

Chemometric models including supervised partial least-squares discriminant analysis and orthogonal partial least-squares discriminant analysis were obtained with MetaboAnalyst 3.0 (Xia and Wishart, 2011) us-

ing log-transformed and autoscaled data. Metabolites with 30% and more of missing data were removed from analysis; the missing values (less than 30%) for a particular metabolite were imputed with the observed minimum detection value, assuming their level was below the instrument detection limit. A probability level of $P < 0.05$ was considered statistically significant. Statistical models were validated by 10-fold cross-validation and response permutation with 500 random reclassifications. A regression coefficient plot with 95% jackknifed confident intervals was used to determine which metabolites drove observed clustering patterns. Parameters R^2Y (linear regression model of predicted and observed variables) and Q^2 (quality assessment statistics) are more than 0.5 indicating a robust model with prominent predictive ability. Metabolites with variable importance for projection (VIP) values exceeding 1.5 were selected as the significantly different metabolites (Storey, 2002; Wiklund et al., 2008; Issaq and Veenstra, 2013).

Western blot data with nonnormal distribution and all mRNA abundance data were $\log_2$ transformed to obtain a normal distribution before statistical analysis. Statistical analysis was performed using the MIXED model in SAS (version 9.3, SAS Institute Inc., Cary, NC) with ratios of Lys to Thr, Ile, Val, and Leu as fixed effects and cell culture as the random effect. The control group in the present study was the same as that in Dong et al. (2018) in which the specific objective was to evaluate the effect of increasing the supply of Met while holding Lys constant on the same gene and protein targets that are reported herein. Treatment means were compared using the PDIF statement in SAS after correction using Tukey's. Significance was declared at a PDIF Tukey-adjusted $P < 0.05$.

RESULTS

Metabolomics

In total 162 compounds were identified and quantified by GC-MS, the list of metabolites is reported in Supplemental Table S4 (<https://doi.org/10.3168/jds.2017-13707>). The partial least-squares discriminant analysis and orthogonal partial least-squares discriminant analysis showed significantly separated clusters between IPAA and LT1.3, LI1.29, LV1.12, and LL0.78 (Supplemental Figures S1 and S2; <https://doi.org/10.3168/jds.2017-13707>). The R^2Y and Q^2 values >0.5 are both indicative of proper reliability of the model. The R^2Y was greater than 0.992 in all comparisons, indicating that the models had good reliability. Most of Q^2 values were at least 0.51 indicating the models had outstanding predictive ability (Supplemental Figures

S1 and S2; <https://doi.org/10.3168/jds.2017-13707>). The top 15 significantly different metabolites between IPAA and LT1.3, LI1.29, LV1.12, and LL0.78 (VIP > 1.5, $P < 0.05$) are depicted in the VIP plots (Figure 1). The red- and green-colored squares denote a high or low concentration.

Compared with IPAA, culture with LT1.3 increased the concentration of certain EAA (Leu, Thr) and the NEAA Glu, Gln, Asp, Gly, and Ser. Compared with IPAA, an increase in availability of Ile (LI1.29) led to higher Leu concentration and concentration of Pro,

Asp, Glu, and Gln. Relative to IPAA, culture with LV1.12 also increased the concentration of various EAA (His, Met, Ile, and Leu) and NEAA (Gln, Glu, Ser, Gly, and Asp).

In addition to changes in AA concentrations, all of the treatment groups (LT1.3, LI1.29, LV1.12, and LL0.78) had greater concentrations of uric acid relative to IPAA. Compared with IPAA, culture with LT1.3 and LV1.12 led to greater concentration of phosphoric acid, but incubation with LI1.29 and LL0.78 decreased the concentration of phosphoric acid. Compared with

Figure 1. Metabolites ranked by variable importance in projection (VIP) analysis in response to culturing mammary epithelial cells with different EAA ratios: IPAA = ideal profile of Lys:Met and fixed ratio of other EAA; LT1.3 = Lys:Thr ratio of 1.3:1; LI1.29 = Lys:Ile ratio of 1.29:1; LV1.12 = Lys:Val ratio of 1.12:1; LL0.78 = Lys:Leu ratio of 0.78:1.

IPAA, culture with LV1.12 and LL0.78 decreased the concentration of glucose-6-phosphate. A lower concentration of pyruvic acid and 3-phosphoglycerate was observed with the supplementation of Ile and Leu relative to IPAA.

mRNA Abundance

The effects of EAA ratios on mRNA abundance of AA transporters and β -casein (*CSN2*) are shown in Table 2. Compared with IPAA, all treatments resulted in greater ($P < 0.01$) mRNA abundance of *SLC3A2*. Compared with IPAA, treatment LL0.78 had greater ($P < 0.05$) mRNA abundance of *SLC36A1*, *SLC38A9*, and *SLC38A2*. Higher ($P < 0.05$) mRNA abundance of *SLC38A9* also was observed with treatment LT1.3 compared with IPAA.

Treatment LV1.12 increased ($P < 0.01$) the mRNA abundance of *SLC38A9* relative to IPAA. However, compared with IPAA, treatment LI1.29 decreased ($P < 0.05$) the mRNA abundance of *SLC7A5*, *SLC38A2*, and *SLC43A1*. Compared with IPAA, a lower mRNA abundance of *SLC43A1* was also observed in treatment LT1.3 and treatment LV1.12. Relative to IPAA, treatment LV1.12 was with the significant trend ($P = 0.15$) to increase mRNA abundance of *CSN2*. However, treatment LI1.29 tended to decrease the mRNA abundance of *CSN2*.

Protein Abundance and Phosphorylation Status of mTOR Pathway

The effects of EAA ratios on protein abundance and phosphorylation status of signaling proteins in the mTOR pathway are reported in Table 3. Compared with IPAA, an increase in both Ile (LI1.29) and Val (LV1.12) resulted in greater ($P < 0.01$) phosphoryla-

tion ratio of mTORC1. However, the increase in Thr (LT1.3) led to lower ($P < 0.01$) phosphorylation ratio of mTORC1. Compared with IPAA, phosphorylation ratio of Akt increased ($P < 0.01$) only in LV (1.12). An increase ($P < 0.05$) in phosphorylation ratio of RPS6 was observed with both Val (1.12) and Leu (LL0.78) compared with IPAA. An increasing trend of S6K1 phosphorylation ratio was also observed in both Val (1.12) and Leu (LL0.78) relative to IPAA. Compared with IPAA, the phosphorylation ratio of 4EBP1 was increased ($P < 0.05$) by LT1.3. Compared with IPAA, the phosphorylation ratio of eIF2 α was decreased ($P < 0.01$) with the supplementary of Ile, Val, and Leu. No significant change was observed in the phosphorylation ratio of eEF2 relative to IPAA.

Promoter Region-Specific DNA Methylation

The effects of EAA ratios on promoter region-specific DNA methylation of mTORC1 and α_{S1} -casein (*CSN1S1*) are reported in Table 4. The degree of methylation of *MTORC1* was below 2%, but that of *CSN1S1* was greater than 80% regardless of treatment. Compared with IPAA, an increase in Ile, Val, and Leu led to greater mRNA abundance ($P < 0.01$) of *CSN1S1*. The LV1.12 has great potential to increase ($P = 0.15$) the methylation of *CSN1S1* relative to IPAA. No significant difference was observed in mRNA abundance and methylation of mTORC1 relative to IPAA.

DISCUSSION

Intracellular Metabolism

Thr, Ser, and Gly Metabolism. Although the exact mechanisms are unknown, in vivo and in vitro research revealed that Thr could promote milk protein

Table 2. Effects of different EAA ratios on mRNA abundance (log-scale) of AA transporters and β -casein (*CSN2*)¹

Gene	Treatment ²					SEM	P-value
	IPAA	LT1.3	LI1.29	LV1.12	LL0.78		
<i>SLC3A2</i>	0.32 ^b	0.63 ^a	0.93 ^a	0.84 ^a	0.77 ^a	0.14	<0.001
<i>SLC7A5</i>	1.14 ^{ab}	1.10 ^b	0.76 ^c	1.11 ^{ab}	1.25 ^a	0.03	<0.001
<i>SLC36A1</i>	1.11 ^b	1.17 ^{ab}	0.98 ^b	1.18 ^{ab}	1.39 ^a	0.06	0.002
<i>SLC38A2</i>	1.26 ^{bc}	1.38 ^{ab}	0.94 ^d	1.17 ^c	1.45 ^a	0.04	<0.001
<i>SLC38A9</i>	1.05 ^b	1.31 ^a	0.91 ^b	1.30 ^a	1.42 ^a	0.05	<0.001
<i>SLC43A1</i>	1.20 ^a	0.99 ^{bc}	0.87 ^c	1.00 ^b	1.23 ^a	0.04	<0.001
<i>CSN2</i>	1.56 ^{ab}	1.58 ^{ab}	1.15 ^b	2.08 ^a	1.67 ^{ab}	0.17	0.006

^{a-d}Means in a row without a common superscript differ, $P < 0.05$.

¹Data are LSM and SEM (n = 5 replicates/treatment).

²IPAA = ideal profile of Lys:Met and fixed ratio of other EAA according to Table 1; LT1.3 = Lys:Thr ratio of 1.3:1; LI1.29 = Lys:Ile ratio of 1.29:1; LV1.12 = Lys:Val ratio of 1.12:1; LL0.78 = Lys:Leu ratio of 0.78:1.

Table 3. Effects of different EAA ratios on protein abundance and phosphorylation status of signaling proteins¹

Item	Treatment ²					SEM	P-value
	IPAA	LT1.3	LI1.29	LV1.12	LL0.78		
Total protein ³							
Akt	6.47	10.49	10.11	3.96	13.24	2.67	0.158
mTORC1	0.15 ^b	1.43 ^a	0.27 ^b	0.11 ^b	0.28 ^b	0.10	<0.001
4EBP1	0.72 ^{bc}	0.57 ^c	1.83 ^{ab}	1.94 ^a	1.26 ^{abc}	0.28	0.006
S6K1	4.14 ^a	2.17 ^b	1.75 ^b	1.91 ^b	1.01 ^b	0.34	<0.001
RPS6	7.38 ^{ab}	11.10 ^a	8.58 ^a	3.61 ^b	3.48 ^b	1.02	<0.001
EIF2 α	1.40 ^d	4.39 ^{cd}	7.29 ^{bc}	12.58 ^a	10.28 ^{ab}	1.03	<0.001
EEF2	0.60	0.61	1.08	0.88	0.85	0.15	0.150
Phosphorylated (p) protein							
p-Akt	0.54	0.94	0.61	0.50	1.35	0.26	0.155
p-mTORC1	1.04 ^c	2.47 ^b	3.41 ^a	2.57 ^b	1.48 ^c	0.14	<0.001
p-4EBP1	1.50 ^b	2.23 ^a	6.40 ^a	6.33 ^a	3.30 ^{ab}	1.05	0.007
p-S6K1	0.24 ^a	0.14 ^{abc}	0.08 ^c	0.18 ^{ab}	0.10 ^{bc}	0.02	<0.001
p-RPS6	1.82 ^b	4.78 ^a	4.39 ^a	4.45 ^a	2.21 ^b	0.38	<0.001
p-EIF2 α	0.76 ^{ab}	0.96 ^a	0.69 ^{ab}	0.5 ^b	0.75 ^{ab}	0.09	0.02
p-EEF2	9.63	14.54	9.76	21.82	16.52	2.94	0.04
Ratio							
p-Akt/Akt	0.08 ^{bc}	0.09 ^{bc}	0.06 ^c	0.14 ^a	0.10 ^b	0.01	<0.001
p-mTORC1/mTORC1	7.18 ^c	1.78 ^d	12.81 ^b	23.98 ^a	5.36 ^{cd}	0.83	<0.001
p-4EBP1/4EBP1	2.11 ^b	4.54 ^a	3.57 ^{ab}	3.44 ^{ab}	2.54 ^{ab}	0.48	0.018
p-S6K1/S6K1	0.06	0.07	0.05	0.09	0.10	0.01	0.026
p-RPS6/RPS6	0.25 ^c	0.47 ^{bc}	0.53 ^{bc}	1.29 ^a	0.61 ^b	0.08	<0.001
p-EIF2 α /EIF2 α	0.55 ^a	0.22 ^{ab}	0.09 ^{bc}	0.04 ^c	0.07 ^c	0.02	<0.001
p-EEF2/EEF2	18.22 ^{ab}	24.55 ^a	9.04 ^b	26.79 ^a	20.21 ^{ab}	3.13	0.006

^{a-d}Means in a row without a common superscript differ, $P < 0.05$.

¹Data are LSM and SEM (n = 5 replicates per treatment).

²IPAA = ideal profile of Lys:Met and fixed ratio of other EAA according to Table 1; LT1.3 = Lys:Thr ratio of 1.3:1; LI1.29 = Lys:Ile ratio of 1.29:1; LV1.12 = Lys:Val ratio of 1.12:1; LL0.78 = Lys:Leu ratio of 0.78:1.

³Akt = serine/threonine kinase B; mTORC1 = mammalian target of rapamycin complex 1; 4EBP1 = eukaryotic initiation factor 4E binding protein 1; S6K1 = ribosomal protein S6 kinase; RPS6 = ribosomal protein S6; EIF2 α = α subunit of eukaryotic translation initiation factor 2; EEF2 = eukaryotic translation elongation factor 2.

synthesis by increasing mTORC1 phosphorylation (Arriola Apelo et al., 2014; Zhou et al., 2015). The fact that Thr uptake by mammary gland is in excess of its output in milk protein (Lapierre et al., 2012) indicates this AA is likely metabolized to other compounds. The enzyme threonine dehydrogenase metabolizes Thr to Gly, and its activity is positively correlated with Thr

concentration (Floc'h et al., 1996; Davis and Austic, 1997; Lee et al., 2014). If metabolism of supplemental Thr enhanced Gly synthesis via threonine dehydrogenase, it could have had a positive effect on regulation of cellular proliferation and the synthesis of Ser as part of the one-carbon metabolism pathway (Han et al., 2013; Tang et al., 2014). It is well-established that Ser/

Table 4. Effects of different EAA ratios on promoter region-specific DNA methylation of mammalian target of rapamycin complex 1 (*mTORC1*) and α _{S1}-casein (*CSN1S1*)¹

Gene	Treatment ²					SEM	P-value
	IPAA	LT1.3	LI1.29	LV1.12	LL0.78		
<i>MTORC1</i>							
mRNA abundance	1.09	1.11	1.04	1.20	1.05	0.06	0.16
% Methylation	1.77	1.96	1.80	1.71	1.56	0.18	0.49
<i>CSN1S1</i>							
mRNA abundance	0.10 ^d	0.19 ^{cd}	0.28 ^{bc}	0.66 ^a	0.59 ^{ab}	0.12	<0.001
% Methylation	82.2	92.2	84.0	92.6	90.6	3.18	0.06

^{a-d}Means in a row without a common superscript differ, $P < 0.05$.

¹Data are LSM and SEM (n = 5 replicates per treatment).

²IPAA = ideal profile of Lys:Met and fixed ratio of other EAA according to Table 1; LT1.3 = Lys:Thr ratio of 1.3:1; LI1.29 = Lys:Ile ratio of 1.29:1; LV1.12 = Lys:Val ratio of 1.12:1; LL0.78 = Lys:Leu ratio of 0.78:1.

Gly contributes to cellular metabolism through the Gly cleavage system, refueling the one-carbon metabolism pathway. Taken together, results suggest that an increase in Thr supply resulting in higher concentration of Thr, Gly, and Ser is an indication that the LT1.3 treatment stimulated Thr-Gly-Ser metabolism; however, the fact that this treatment did not upregulate *CSN2* and *CSN1S1*, and actually decreased phosphorylation of mTORC1 seems to suggest a limited capacity for enhancing milk protein synthesis beyond what can be achieved with IPAA. We speculate that the lack of response to Thr was due to its limited intramammary catabolism (Lapierre et al., 2012),

Branched-Chain AA Metabolism. Although Ile and Leu have been demonstrated to independently regulate the activity of mTORC1 and protein synthesis in bovine mammary cells (BMEC; Appuhamy et al., 2012); to our knowledge, there are no reports on the effect of ratio of Lys to Leu or Lys to Ile on the regulation of milk protein synthesis. Furthermore, compared with a theoretically optimal AA mixture, decreasing Val relative to Lys reduced β -casein mRNA abundance (Li et al., 2017). Therefore, an important objective in the present study was to evaluate the individual effects of the branched-chain amino acids (BCAA).

The BCAA are typically taken up by mammary gland in excess of the amount secreted into milk (Derrig et al., 1974; Clark et al., 1977; Wohlt et al., 1977). The excess BCAA are mostly degraded to intermediary metabolites that could indirectly regulate protein synthesis (Zhang, 2017). Therefore, it is plausible that even after supplementation with Ile, Val, or Leu their concentrations are not significantly increased, except for an increase in Leu in response to LI1.29 and LV1.12 (also LT1.3), which was the case in the present study.

Work with bovine mammary tissue revealed that the BCAA can be metabolized to a small but significant extent to Glu and Asp in addition to β -OH-isobutyrate (with Val), isovalerate (with Leu), and methylmalonate (with Ile; Wohlt et al., 1977). Other metabolites produced during incubation with Val included α -ketoisovalerate and isobutyrate. Incubation with Leu also resulted in production of α -ketoisocaproate, acetate, and citrate. Among the BCAA, incubation with Ile led to the smallest concentrations of α -keto- β -methylvalerate, propionate, and acetate (Wohlt et al., 1977).

Clearly, the current GC-MS approach allowed for higher resolution in terms of intermediate metabolites produced during the metabolism of BCAA. Although it is not intuitive how most of the top metabolites identified with increasing supplementation of Val could play a role in milk protein synthesis regulation, the LV1.12 treatment in this study was associated with the high-

est phosphorylation of mTORC1, Akt, and RPS6, and upregulation of *CSN2* and *CSN1S1*. Based on previous data, we speculate that Val could regulate mTORC1 activity through Rag proteins (related small guanosine triphosphatases), which function to enhance the interaction of mTORC1 with its activator Rheb proteins (Ras homolog enriched in brain; Sancak et al., 2008).

Gln and Glu Metabolism. Glutamine and Glu are at the center of amino nitrogen interchanges and hence play important roles in AA metabolism (Kelly and Stanley, 2001). These NEAA are quantitatively the most abundant in milk protein (Derrig et al., 1974). Because of the markedly low uptake by the mammary gland relative to secretion in milk protein, it is thought that Gln and Glu are synthesized by the mammary gland (Guinard et al., 1994). Activity of glutaminase and glutamine synthetase catalyze the interconversion of Gln to Glu in BMEC (Manjarin et al., 2014), but the importance of Gln is underscored by the fact that Glu cannot easily be transported across plasma membranes (Newsholme et al., 2003). In addition, bi-directional flux of Gln across cellular plasma membranes (via SLC7A5/SLC3A2) in response to EAA availability (and Leu in particular) appears to be a key factor in the activation of mTORC1 (Nicklin et al., 2009). The high concentration of Gln in response to LT1.3, LI1.29, and LV1.12 agrees with the greater concentration of Leu suggesting a similar mechanism of control of mTORC1 in mammary cells. Furthermore, the high VIP score for Glu in response to LV1.12 agrees with the highest phosphorylation of mTORC1 and abundance of *CSN2* and *CSN1S1*.

The high concentration of Glu observed in response to LT1.3, LI1.29, and LV1.12 indicated a higher degree of AA utilization through reducing AA oxidation, a response that might have an effect on milk protein synthesis. Compared with Gln, Glu appears to be more important in terms of oxidation because it can directly donate or receive amino groups. Glutamate dehydrogenase and amino transferase are important in the interconversion of Glu and 2-oxoglutarate (Brosnan and Brosnan, 2013). In fact, it is well known that 2-oxoglutarate is an intermediate in the catabolism of most AA and can spare EAA from oxidation via reamination with Glu to form other AA (Lobley et al., 2001). Most EAA are potential oxo-acid acceptors, and in particular Met and Phe are confirmed as the preferred acceptors of 2-oxoglutarate (Blarzino et al., 1994).

Met and His Metabolism. Methionine and His are both considered as limiting AA partly because deficiencies of Met and His could decrease the availability of other EAA (Kim et al., 1999; Manjarin et al., 2014). The higher intracellular concentration of Met and His in response to LV1.12 indicated that an increase in Val

based on the IPAA might further promote the uptake of EAA such as Met and His. It has been demonstrated that enhancing the postruminal supply of Met, Lys, and His could promote milk protein synthesis (Giallongo et al., 2016). Furthermore, at least in vitro, His up-regulated milk protein gene expression partly through increasing the activity of the mTOR pathway (Gao et al., 2015). Therefore, it is reasonable to speculate that a higher intracellular concentration of both Met and His when the supplementation of Val increases may have a positive effect on milk protein synthesis. Because the AA transporters examined did not uniquely respond to an increase in Val availability, the mechanism whereby this AA might stimulate the preferential uptake of Met and His is difficult to explain. Although we did not detect an increase in histamine, it is possible that the increase in His in response to LV1.12 would indirectly affect the growth of BMEC (Davio et al., 1994) through the conversion of His to histamine. It also is possible that an increase in His would affect flux through the histidine/histamine/S-adenosylmethionine/*N*-methylhistamine pathway and elicit an effect on methyl group metabolism (Rai et al., 2014).

Arg Metabolism. Although there was no significant change in Arg concentration across treatments, the increase in concentration of *N*-acetylglutamate in response to enhanced availability of Thr, Ile, and Val was suggestive of potential alterations in Arg metabolism. Activity of carbamoylphosphate synthetase I, the rate-limiting enzyme for Arg synthesis in the urea cycle, is allosterically activated by *N*-acetylglutamate (Grisolia and Cohen, 1953). The fact that we did not detect increases in urea, ornithine, or putrescine in response to LT1.3, LI1.29, and LV1.12 but detected a lower intracellular concentration of putrescine in LL0.78 seems to suggest that an increase in Thr, Ile, Val, and Leu relative to IPAA might favor the utilization of Arg for milk protein synthesis via different mechanisms. For instance, the fact that putrescine (an intermediate metabolite derived from the arginine-ornithine-putrescine metabolism pathway) could promote milk protein synthesis in BMEC (Wang et al., 2017) might partly explain the lack of increase in mTORC1 phosphorylation in response to LL0.78. However, the fact that various AA transporters (*SLC3A2*, *SLC7A5*, *SLC36A1*, *SLC38A2*, *SLC38A9*) and *CSN1S1* were upregulated with LL0.78 compared with IPAA seems to suggest that increased Leu availability could regulate milk protein synthesis by different mechanisms. Another possibility given the lack of response in mTORC1 phosphorylation in response to LL0.78 compared with IPAA is that the increase in Leu created an imbalance of EAA.

AA Transport

In the present experiment, we measured transcription of 6 AA transporters (*SLC3A2*, *SLC7A5*, *SLC36A1*, *SLC38A2*, *SLC38A9*, and *SLC43A1*) associated with mTORC1 activity and the regulation of AA transport (Heublein et al., 2010; Fotiadis et al., 2013; Broer, 2014). The *SLC3A2* and *SLC7A5* constitute heteromeric amino acid transporters (**HAT**), responsible for the transport of various types of AA (Palacin and Kanai, 2004). The *SLC7A5* functions as the transport competent subunit of the HAT complex (Napolitano et al., 2015), whereas *SLC3A2* is responsible for the localization of HAT to the plasma membrane and does not pump AA into or out of cells (Wang et al., 2012; Napolitano et al., 2015). Therefore, we speculate that the upregulation of *SLC3A2* due to the increase in Thr, Ile, Val, and Leu relative to IPAA could promote AA transport and direct the HAT to the cell membrane. Despite the lack of effect on mTORC1 phosphorylation, in contrast to other treatments, the upregulation of *SLC36A1*, *SLC38A2*, and *SLC38A9* in response to LL0.78 was suggestive of some specificity toward Leu. From a mechanistic standpoint, the lack of significant change in intracellular concentration of Gln when the supply of Leu increased could be a reason for the lack of response on mTORC1. Previous data indicated that by promoting the influx of Leu through a bidirectional transport mechanism, Gln can activate mTORC1 (Zheng et al., 2016). A deficiency in Gln supply could trigger the uptake of Leu (Chen et al., 2014); thus, we speculate that in an AA-sufficient state there is a different mechanism whereby Leu can regulate mTOR pathway activity.

The protein *SLC38A9* is a new member of the *SLC38* transporter family that plays a role in the pumping of neutral AA out of the cell (Broer, 2014). Recent studies have reported that *SLC38A9* could indirectly participate in the regulation of mTORC1 activation as a component of the regulator-RAG GTPase machinery (Rebsamen et al., 2015; Wang et al., 2015). To our knowledge, no data have been published linking EAA ratios and *SLC38A9* activity in the context of milk protein synthesis in BMEC.

Overall, data from the present study indicate that an increase in Thr, Val, and Leu could affect the activation of mTORC1 and milk protein synthesis potentially through the regulation of AA transporter abundance. However, because a low protein or AA level could also induce the increase in mRNA abundance of AA transporters (Karasov et al., 1987; Wang et al., 2017), it is necessary to further test whether these treatments

with an increase in Val and Ile based on the IPAA could enhance the activity of mTOR pathway and milk protein synthesis.

mTOR Signaling Pathway Regulation

The activity of the mTOR pathway is partly under the regulation of EAA availability (Proud, 2004). The mTORC1, as a protein kinase, is at the core of a signaling pathway that regulates protein synthesis by integrating intracellular AA availability and endocrine signals from insulin and some growth factors (Veverka et al., 2008). The present data underscore that greater availability of Ile and Val when the ratio of Lys:Met is ideal increased phosphorylation of mTORC1, indicating these AA could further stimulate milk protein synthesis. As a whole, however, increasing Val was most important in terms of phosphorylation of mTORC1 and Akt, and upregulation of various AA transporters and casein genes.

It is noteworthy that, unlike Ile and Val, treatment with LT1.3 led to lower phosphorylation ratio of Akt and mTORC1, which could have resulted from feedback inhibition of the IRS1/Akt/mTOR pathway. It has been demonstrated that rapamycin, an inhibitor of mTORC1, could increase the mRNA abundance of *IRS1* and promote the phosphorylation of Akt (O'Reilly et al., 2006). Because Akt activation is mainly induced by insulin and the same dosage of insulin was used for all treatments, the data suggest that Val (in particular) could regulate Akt phosphorylation status potentially through sensitizing the response of Akt to insulin. Although there is some evidence that insulin regulates the activity of Akt independent of AA (Appuhamy et al., 2011a), the response to Val was similar to previous work in bovine mammary epithelial cells (Burgos et al., 2010). Further studies will have to be performed to better understand the linkages between insulin and EAA.

The proteins 4EBP1 and S6K1 serve as parallel regulators of mRNA translation (Ruvinsky and Meyuhas, 2006). The 4EBP1 mediates the formation of eIF4F to promote the translation initiation by releasing the phosphorylated eIF4E (Proud, 2004; Avruch et al., 2009). The greater phosphorylation status of 4EBP1 in response to LT1.3 and LI1.29 suggested that increasing Thr and Ile supplementation might promote translation initiation. Our findings are consistent with previous research in mouse mammary cells (Liu et al., 2017).

In general, there is a positive association between phosphorylation status of mTORC1 and 4EBP1 as was detected with LI1.29. The opposite was observed with LT1.3. Based on data indicating that only rapamycin and Akt inhibitors used together can markedly decrease the phosphorylation of 4EBP1 (Mi et

al., 2015), we speculate that a feedback mechanism exists in the mTORC1–4EBP1 signaling pathway. The exact mechanisms for these responses will need further research. Along with the greater mTORC1 phosphorylation, the tendency for greater 4EBP1 phosphorylation in response to LV1.12 underscored the potential for Val to affect milk protein synthesis partly through the mTORC1–4EBP1 signaling pathway. A similar response was reported in a study with mammary tissue slices and porcine trophectoderm cells (Kong et al., 2012; Appuhamy et al., 2014).

Phosphorylation of S6K1 also is associated with mTORC1 activity (Appuhamy et al., 2012). It has been demonstrated that active S6K1 could activate eIF4B and eIF3 and inactivate PDCD4 to promote translation initiation via phosphorylation (Xie and Proud, 2014). Therefore, the great trend to increase phosphorylation of S6K1 with LV1.12 and LL0.78 is further indication that increasing availability of Val and Leu could positively affect mRNA translation.

Both eEF2 and RPS6 are downstream targets of S6K1. When dephosphorylated, eEF2 promotes mRNA translation elongation (Proud, 2004). Previous studies demonstrated an inverse association between the phosphorylation status of eEF2 and mTORC1 in BMEC (Appuhamy et al., 2011a). Except for LI1.29, in the present study, no significant difference in eEF2 phosphorylation was observed in response to LT1.3, LV1.12, and LL0.78 despite the marked increase in phosphorylation with LV1.12. These responses suggested that other factors might play a role in the regulation of eEF2 phosphorylation in addition to EAA signaling, and particularly the supplementation of Val.

A previous study demonstrated that RPS6 could be activated by the mTORC1/S6K1 pathway and stimulate mRNA translation and overall protein synthesis (Jefferies et al., 1997). In the present study, the higher RPS6 phosphorylation status with LV1.12 and LL0.78 compared with IPAA was consistent with prior experiments in human HEK293 cells, rat L6 myoblasts and BMEC (Kimball et al., 1999; Tang et al., 2001; Appuhamy et al., 2012). Although Leu also significantly increased phosphorylation of RPS6, the fact that Val resulted in a 5-fold increase in phosphorylation of RPS6 along with marked increases in mTORC1 and Akt phosphorylation underscore the uniqueness of this EAA in the control of protein synthesis in mammary cells.

The eIF2 α plays a critical role for the initiation of mRNA translation by mediating the recruitment of the Met-tRNA to the 40S ribosomal subunits (Appuhamy et al., 2012). Phosphorylated eIF2 α could decrease the binding efficiency of Met-tRNA to the 40S ribosomal subunits (Kimball, 1999). In a previous study, a jugular infusion of EAA reduced eIF2 α phosphorylation

in mammary tissue of lactating cows (Toerien et al., 2010). In contrast, AA deprivation resulted in higher phosphorylation of eIF2 α in mouse liver and skeletal muscle (Anthony et al., 2004). Therefore, taking into account the upregulation of *CSN2* and *CSN1S1* along with increases in phosphorylation of Akt, mTORC1, and RPS6, the downregulation of eIF2 α phosphorylation with LV1.12 would have provided optimal conditions to stimulate protein synthesis beyond incubation with IPAA.

Methylation

A previous in vivo study demonstrated that *CSN1S1* abundance could be regulated in part by the methylation status in response to environmental factors, milking frequency, and stage of lactation (Nguyen et al., 2014). The greater percentage of *CSN1S1* methylation induced by LV1.12 along with the upregulation of *CSN1S1* indicate that this EAA can alter methylation status without causing its downregulation or that the CpG sites evaluated might have less effect on the final transcription of *CSN1S1*. It also should be noted that our data pertain to CpG regions in the promoter rather than a distal upstream region as measured by Nguyen et al. (2014); thus, the data are not directly comparable between studies. In addition, because the MAC-T cell line does not secrete protein, the changes observed at the mRNA abundance level might not be directly extrapolated to an in vivo condition. However, given that LV1.12 caused changes at the mRNA, protein, and metabolite level indicating a positive effect on milk protein synthesis, it could be speculated that these responses are biologically relevant. Clearly, additional experiments are needed to clarify these results.

CONCLUSIONS

Our research provided more systematic evidence based on metabolomics that EAA ratios could affect protein synthesis in part by regulating intracellular metabolism. An increase in availability of Val relative to Lys (Lys:Val, 1.12:1) when Lys:Met is ideal could further stimulate milk protein synthesis by optimizing the profile of intracellular EAA, enhancing the activity of mTORC1, upregulating mRNA abundance of AA transporters, β -casein, and α_{S1} -casein and methylation of α_{S1} -casein. Future studies should be performed to verify these responses in vivo.

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