RESEARCH ARTICLE

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D-Methionine and 2-hydroxy-4-methylthiobutanoic acid i alter beta-casein, proteins and metabolites linked in milk protein synthesis in bovine mammary epithelial cells

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Abstract

This study aims to determine the effects of D-methionine (D-Met) isomer and the methionine precursor 2-hydroxy-4-methylthiobutanoic acid i (HMBi) supplementation on milk protein synthesis on immortalized bovine mammary epithelial cell (MAC-T). MAC-T cells were seeded using 10-cm dishes and cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) basic medium. The basic medium of DMEM/F12 was replaced with the lactogenic DMEM/F12 differentiation medium when 90% of MAC-T cells reached confluency. The best dosage at 0.6 mM of D-Met and HMBi and incubation time at 72 h were used uniformly for all treatments. Each treatment was replicated six times wherein treatments were randomly assigned in a 6-well plate. Cell, medium, and total protein were determined using a bicinchoninic acid protein assay kit. Genes, proteomics and metabolomics analyses were also done to determine the mechanism of the milk protein synthesis pathway. Data were analyzed by two-way analysis of variance (ANOVA) with supplement type and plate as fixed effects. The least significant difference test was used to evaluate the differences among treatments. The HMBi treatment group had the highest beta-casein and S6 kinase beta-1 (S6K1) mRNA gene expression levels. HMBi and D-Met treatments have higher gene expressions compared to the control group. In terms of medium protein content, HMBi had a higher medium protein quantity than the control although not significantly different from the D-Met group. HMBi supplementation stimulated the production of eukaryotic translation initiation factor 3 subunit protein essential for protein translation initiation resulting in higher medium protein synthesis in the HMBi group than in the control group. The protein pathway analysis results showed that the D-Met group stimulated fructose–galactose metabolism, glycolysis pathway, phosphoinositide 3 kinase, and pyruvate metabolism. The HMBi group stimulated the pentose phosphate and glycolysis pathways. Metabolite analysis revealed that the D-Met treatment group increased seven metabolites and decreased uridine monophosphate (UMP) production. HMBi

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Competing interests
No potential conflict of interest relevant to this article was reported.

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Availability of data and material
Upon a reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Jeon SW, Conejos JRV, Lee HG.
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supplementation increased the production of three metabolites and decreased UMP and N-acetyl-L-glutamate production. Taken together, D-Met and HMBi supplementation are effective in stimulating milk protein synthesis in MAC-T cells by genes, proteins, and metabolites stimulation linked to milk protein synthesis.

Keywords: D-Methionine (D-met), 2-Hydroxy-4-methylthiobutanoic acid i (HMBi), Immortalized bovine mammary epithelial cell line (MAC-T), Methionine, Milk protein, Proteomics

INTRODUCTION

In many feed systems, methionine (Met) is known as a limiting amino acid (AA) for milk protein synthesis in dairy cows [1–3]. Met plays a critical role in protein synthesis and is active in many other biochemical and cellular processes [2,3]. The primary role of Met is to initiate mRNA translation by binding methionyl-tRNA into the 40S ribosome, then joining with the 60S ribosome to form the 80S ribosome [4]. Met supplementation has been reported to increase protein expression, mammalian target of rapamycin (mTOR) phosphorylation, and beta-casein expression [5]. Supplementation with Met in bovine mammary epithelial cells causes the increase of mTOR pathway activation and phosphorylation of glycogen synthase kinase 3 which acts as an inhibitor of mTOR [6]. Met supplementation in bovine mammary epithelial resulted in positive effects on milk protein synthesis because of phosphorylation of proteins related to the mTOR pathway [7]. Met infusion into bovine mammary tissue has also been reported to increase S6 kinase beta-1 (S6K1) and ribosomal protein (RP)S6 phosphorylation [8]. Several past studies have shown that nutrient–gene interaction allows Met to directly regulate the expression of genes linked to milk protein synthesis [9,10].

D-Met is one form of Met that can be made synthetically and used as a supplement in the diets of dairy cows. The roles of enzymes are particular to the specific isomer. D-Met is converted into L-form via a two-step process, mainly in the liver, kidney, and other tissues [11–13]. Amino acid (AA) oxidase removes the amine group via an oxidation reaction to form the intermediate 2-keto-4-methylthiobutanoic, followed by attachment of transaminase to the amine group forming the L-Met [11–13].

The Met hydroxy analog 2-hydroxy-4-methylthiobutanoic acid (HMBi), a Met pre-cursor, has long been proposed to provide Met and thus increase the milk protein contents in dairy cows fed with Met-limited ration [14]. The chemical structure of HMBi is mainly similar to that of Met. The main difference in their structures is that instead of an amine group, HMBi has a hydroxyl group (OH). The analog also exists in both D- and L- isomer forms. HMBi has several beneficial effects on rumen fermentation, including enhancing cellulolytic activity, fiber degradation, and increased acetate production [15–17].

Recently, different omics studies detected distinct clusters of differentially concentrated metabolites following treatment with various ratios of Met, Lysine, Phenylalanine, Valine, and Tryptophan [18–20]. However, to our knowledge, no studies have applied proteomic and metabolomic approaches to analyze the effects of supplementation with D-Met and its precursor HMBi on the beta-casein synthesis and milk protein synthesis-related pathways. The present study aims to determine the effect of adding D-Met and the Met precursor HMBi on the stimulation of milk protein synthesis in bovine mammary epithelial cells. It also investigates the related pathways that increase protein synthesis and beta-casein expression in immortalized bovine mammary epithelial cell lin (MAC-T) cells using proteomic and metabolomic data.

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MATERIALS AND METHODS

Amino acid dosage and time of sampling

MAC-T cells which are immortalized mammary epithelial [21] came from McGill University, Canada, and were cultured following the procedure of Wang et al. [21,22]. The AA profile of the differentiation medium is shown in Table 1. Cell confluency can be estimated by considering the doubling time characteristics of our cells.

An initial experiment was done to determine the effects of various dosages of supplemental Met and its culture time on beta-casein mRNA expression. Additional Met (+0.3, +0.6, +0.9 mM) were supplemented to lactogenic medium without fetal bovine serum. Cells were harvested 24 hours.

Beta-casein secretion on MAC-T cells was harvested at 0, 12, 24, 36, 48, 72, 96, and 120 h after incubating with the lactogenic medium to determine the effect of the treatments on culture time. Preliminary study shows that 0.6 mM and incubation time at 72 h were the best dosage and incubation time respectively and were used uniformly for all treatments. D-Met and HMBi were obtained from Sigma-Aldrich (M9375) (Sigma-Aldrich, St. Louis, MO, USA) and Adisseo MetaSmart® (Adisseo, Antony, France), respectively. Treatments were replicated six times and randomly assigned on a 6-well plate.

RNA extraction and cDNA synthesis

Using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), the total RNA from MAC-T cells were extracted. The RNA quality and quantity were determined in NanoDrop 1000 spectrophotometer using RNA-40 module (Thermo Fisher, Waltham, MA, USA). Values of > 1.8 A260/280 were acceptable. The cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). The sample was incubated first at 25℃ for 5 min, then 42℃ for 30 min, and finally at 85℃ for 5 min. The cDNA quantity and quality were evaluated using the ssDNA-33 module of the Thermo NanoDrop 1000 spectrophotometer (Thermo Fisher).

Real-time polymerase chain reaction

RT-PCR was done with a total volume of 20 μL/well using 96-micro-well plates (Sigma-Aldrich).

Table 1. Amino acid profile of lactogenic medium (mM)

Amino acid	Lactogenic medium (mM)
Arginine	0.7
Cysteine	0.1
Glutamine	2.5
Glycine	0.25
Histidine	0.15
Isoleucine	0.42
Leucine	0.45
Lysine	0.5
Methionine	0.12
Phenylalanine	0.22
Serine	0.25
Threonine	0.45
Tryptophan	0.04
Tyrosine	0.21
Valine	0.45

Cycling temperature was performed using the T100 Thermal Cycler System (Bio-Rad). Beta-actin served as the reference gene. The reaction mixture is shown in Table 2. RT-PCR oligonucleotide sequences of forward and reverse primers specific for the target genes that were tested and validated are shown in Table 3. RT-PCR reactions were found in Table 4. The Livak method was used to determine the relative gene expression of the target gene to the control group using the threshold cycle ($2^{-\Delta\Delta CT}$ method) [23] to analyze relative gene expression changes from real-time quantitative PCR experiments. Relative quantification of expression levels of target genes in the treatment group was compared to the untreated group. A Gene expression ratio equal to 1 was used for all the control values. Therefore, a relative gene expression value greater or less than 1 correspond to a fold increase or decrease in expression relative to the control. Furthermore, to test the significance of the differences observed, least significant difference (LSD) test was performed.

The melting curve was also considered to determine the Ct. This is to keep the cycle threshold firmly within the geometric phase. This is very important to calculate the fold changes from sample to sample and to get quantity information from a standard curve. Certain regions of the melting curve were also avoided, specifically, Ct was set not to be too low to avoid the noise during amplification. Also, too high Ct was also avoided wherein it is already linear or in the plateau phase of amplification where data are less predictable. We target the spot where all the curves are straight and parallel to one another which is the point where the precision of our replicates is the highest. It is somewhere toward the middle of the geometric phase or maybe slightly higher.

Table 2. Reaction mixture in RT-PCR reaction

Component	Amount
cDNA	50 ng
Forward primer	0.6 μL
Reverse primer	0.6 μL
PCR master mix	10 μL
(DEPC)-treated water	6.3 μL

RT-PCR, real-time polymerase chain reaction; DEPC, diethyl pyrocarbonate.

Table 3. Primer pairs, accession number, annealing temperature, and product sizes of primers used in RT-PCR reaction

Gene	F/R	Sequence (5' → 3')	Accession number	Annealing temperature	Length
Beta-casein	Forward	AAATCTGCACCTTCCTCTGC	XM_015471671.2	59.0℃	123
	Reverse	GAACAGGCAGGACTTTGGAC			
S6K1	Forward	GGACATGGCAGGGGTGTTT	XM_027519044.1	55.6℃	138
	Reverse	GGTATTTGCTCCTGTTACTT			
Beta-actin	Forward	GCATGGAATCCTGCGGC	NM_173979	58.0℃	121
	Reverse	GTAGAGGTCCTTGCGGATGT			

RT-PCR, real-time polymerase chain reaction; S6K1, S6 kinase beta-1.

Table 4. PCR cycling conditions were used to analyze beta-casein expression

PCR cycling step	Temperature	Time	Cycle
Initial incubation	95℃	3 min	1
Denaturation	95℃	10 sec	50
Annealing	55℃	10 sec	1
Extension	72℃	30 sec	1

PCR, polymerase chain reaction.

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Protein extraction and quantification

After 72 h of incubation, the culture medium was collected for protein quantification by centrifuging at 300×g for 5 min at 4℃ by removing the dead cell fragments. The medium was quantified using a Pierce bicinchoninic acid assay (BCA) kit (Pierce Biotechnology, Rockford, IL, USA). The cells attached at the bottom of the well were then washed with 1× phosphate-buffered saline (PBS). Then 200 μL cell lysis buffer (pH 8.3) containing 10 mM Tris/HCl, 8 M urea, 5 mM ethylenediaminetetraacetic acid (EDTA), 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), and 1× protease inhibitor cocktail (GE Healthcare, Piscataway, NJ, USA) were added. Cell lysates were stood at 20℃ for 30 min and centrifuged at 14,000×g for 30 min at 20℃ [21]. The collected cells underwent cell protein quantification by BCA using the Pierce Protein Assay Kit (Pierce Biotechnology).

Proteome analysis

Lysis buffer containing 20 mM Tris, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and complete EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) was used to extract cell proteins. Cell lysates were incubated at 4℃ for 30 min and then centrifuged at 13,000×g for 10 min at 4℃ [24]. All treatments were replicated three times.

The proteome analysis was performed according to the procedures described by Ishihama et al. [24]. The exponentially modified protein abundance index (emPAI)-based abundances compared with the actual values were within 63% on average, which is similar or better than the determination of abundance by protein staining [24]. Also, metabolite quantity determination was done as described by a previous study done by Park et al. [25].

Statistical analysis

Statistical analyses were done using SAS v. 9.1 software (SAS Institute, Cary, NC, USA). The dose responses were analyzed by computing the polynomial orthogonal contrasts (linear, quadratic, cubic) of responses to the supplementation of Met. Data were analyzed by two-way analysis of variance (ANOVA) with supplement type and plate as fixed effects. Significant differences among treatments were evaluated using LSD test. The significant difference was determined at a level of $p < 0.05$. The experimental model was:

$$X_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ijk},$$

Where: μ = grand mean

α_i = effect of Met type and the Met precursor HMBi

β_j = effect of plate

γ_{ij} = interaction of treatment (D-Met/HMBi) and plate

ε_{ijk}= error variability

Significant increases and decreases in protein quantity were determined by the semi-quantification relative ratio (the ratio was > 2 or < 0.5 respectively) [26,27]. The detected proteins were analyzed for pathway analysis (*Bos taurus*) by using a website program (http://www.pantherdb.org accessed on 26 October 2021). Differentially expressed metabolites and affected pathway detection metabolites were analyzed using Dunnett's Multiple Comparison test by SAS software (V 9.1, SAS Institute). Pathway detection metabolites affected by the application of treatments were analyzed using the Metabo Analyst 3.0 program (University of California, Davis), a pathway detection metabolite tool for pathway analysis of cattle (*Bos taurus*).

RESULTS AND DISCUSSION

Amino acid dosage and sampling time

The preliminary Met dosage test showed that +0.6 mM was the optimal concentration for increasing beta-casein mRNA gene expression. Beta-casein mRNA expression increased at a Met-dose of +0.6 mM (Fig. 1). Polynomial orthogonal contrasts (linear, quadratic, cubic) analyzed the dose responses to Met supplementation (Table 5). Linear contrasts of dose responses revealed significant differences ($p < 0.05$).

A cell viability test was performed to evaluate D-Met isomer and HMBi supplementation; the 0.6-mM dose had no significant toxic effect than the control (Fig. 2). The result implies that +0.6 mM is the most effective level for increasing beta-casein mRNA expression identical to the previously reported result [27–29].

The optimal incubation time for increasing beta-casein mRNA expression was 72 h (Fig. 3A; $p < 0.05$). No significant increase in beta-casein mRNA expression was observed even after 72 h. Maximum cell protein synthesis coincides with maximum beta-casein mRNA expression (Fig. 3B; $p < 0.05$). This result indicates that 72 h should be used as the optimal incubation time for further tests of Met isomer and precursor efficacy since MAC-T cells were completely differentiated into beta-casein-secreting cells. Previous studies have reported that relative gene expression for beta-casein production is generally increased by supplementation with Met [28,29].

No significant difference in cell protein upon addition of the treatments (Fig. 4A; $p > 0.05$). The culture medium treated with HMBi led to significantly higher protein content than the control, yet it was not significantly different from the D-Met treatment (Fig. 4B; $p > 0.05$). Availability of Met is critical for regulating translation and a major limiting factor for protein synthesis in the mammary epithelial cells [30–32].

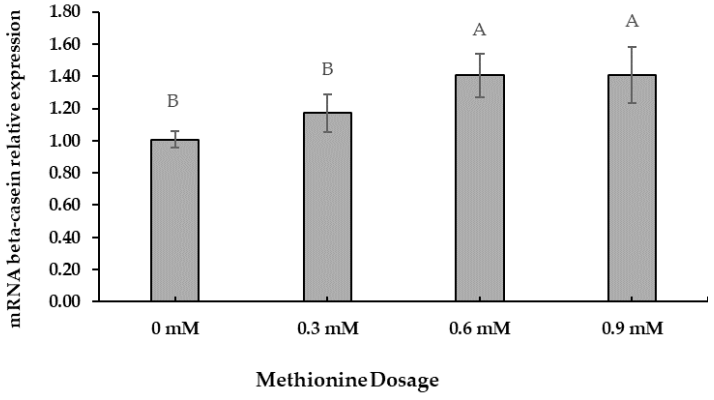

Fig. 1. Beta-casein gene expression level in MAC-T cells supplemented with different levels of Met (0, 0.3, 0.6, 0.9 mM) for 24 h. The values are expressed as means + SE (n = 6) and A, B imply significant differences at $p < 0.05$ by least significant difference (LSD) test. MAC-T, immortalized bovine mammary epithelial cell line; Met, methionine.

Table 5. Polynomial orthogonal contrasts of dose response to methionine supplementation

Contrast	DF	Contrast SS	Mean square	*F*-value	Pr > *F*
Linear	1	0.619	0.619	6.20	0.022
Quadratic	1	0.039	0.039	0.39	0.538
Cubic	1	0.027	0.027	0.27	0.608

DF, degrees of freedom; SS, sum of squares.

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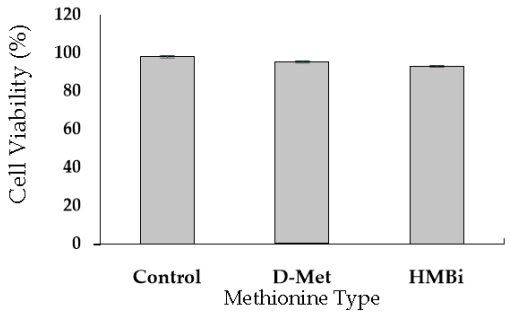

Fig. 2. Cell viability test for D-Met and HMBi supplementation (0.6 mM/L). The values are expressed as means + SE (n = 6). D-Met, D-methionine; HMBi, 2-hydroxy-4-methylthiobutanoic acid i.

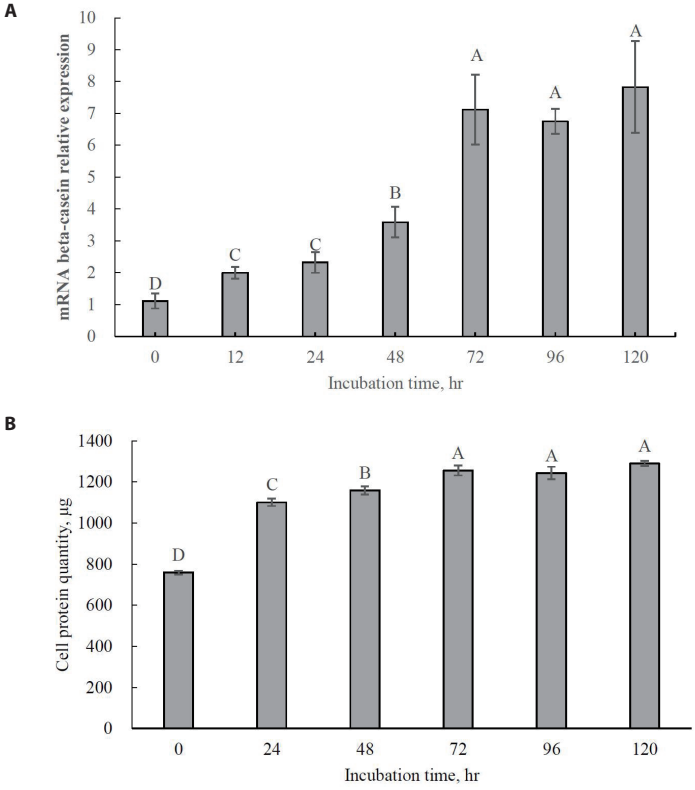

Fig. 3. (A) Beta-casein mRNA expression level; (B) cell protein quantity in MAC-T cells at different time points incubated with methionine (0, 24, 48, 72, 96, 120 h). The values are expressed as means ± SE (n = 6) and A, B, C, D imply significant differences at $p < 0.05$ by least significant difference (LSD) test. MAC-T, immortalized bovine mammary epithelial cell line.

Overall, there were no significant differences in total protein (cell and medium) among the treatments (Fig. 4C; $p > 0.05$). Following supplementation with D -Met and the Met precursor HMBi at optimal dosage and time, HMBi has the highest beta-casein expression (Fig. 4D; $p > 0.05$). The addition of HMBi showed the highest gene expression for S6K1, a downstream target of mTOR pathway (Fig. 4E; $p > 0.05$) [2,7,8].

Proteome analysis

For the proteomics analysis, the upregulated and downregulated proteins in the D-Met group were

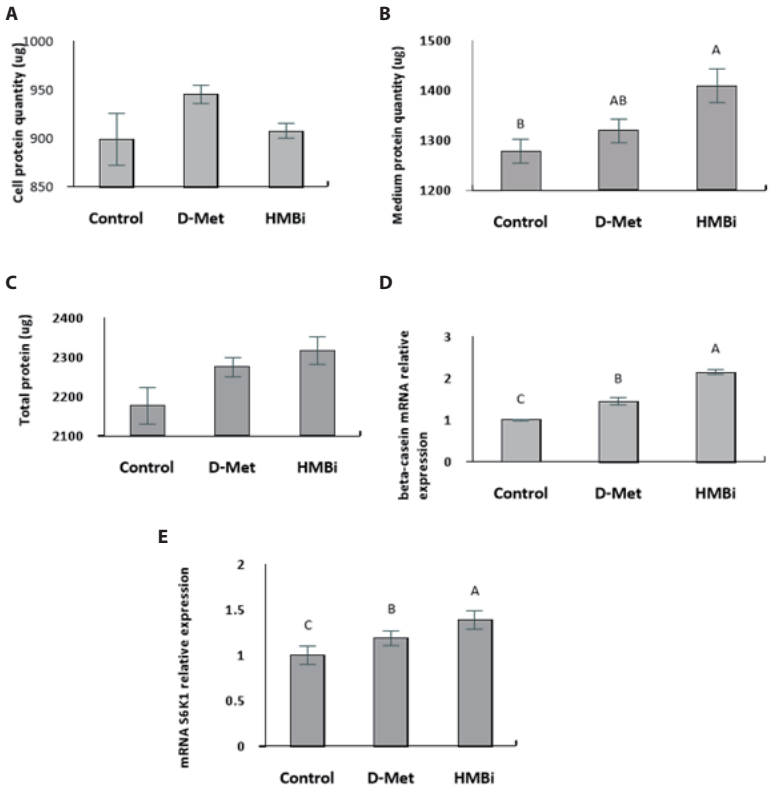

Fig. 4. (A) Cell protein quantity; (B) medium protein quantity; (C) total protein quantity (cell and medium; (D) beta-casein mRNA expression; (E) *S6K1* mRNA expression in MAC-T cells supplemented with D-Methionine (D-Met; +0.6 mM), or 2-hydroxy-4-methylthiobutanoic acid I (HMBi; +0.6 mM). The values are means ± SE (n = 6) and A, B, C imply significant differences at $p < 0.05$ by Least Significant Difference (LSD) test. *S6K1*, S6 kinase beta-1; MAC-T, immortalized bovine mammary epithelial cell line; HMBi, 2-hydroxy-4-methylthiobutanoic acid i.

46 and 68, and 40 and 78 in the HMBi group (Table 6). Expression of proteins linked to protein and energy metabolism was altered after supplementations with D-Met and HMBi. The addition of HMBi upregulated the elongation protein eukaryotic translation initiation factor 3 subunit (EIF3A) but downregulated the ribosomal proteins RPS12 and RPS21. Supplementation with D-Met stimulated phosphoinositide 3 (PI3) kinase, an upstream mTOR activator [19,28,33,34]. The list of all upregulated and downregulated proteins are listed in Tables 8 and 9.

HMBi supplementation stimulated the production of EIF3A protein essential for protein translation initiation [19,28] higher than the D-Met group. EIF3A is vital for stimulating protein synthesis [19,28]. The RPS12 and RPS21 were decreased in the HMBi group but not in the D-Met group. The RPS12 and RPS21 proteins are ribosome protein components found in 40S subunit [35]. Increase in EIF3A and the decrease in RPS12 and RPS21 ribosomal protein components when milk protein synthesis is increasing agreed with previous studies [36,37]. It is said that protein export and transformation of AA going to tRNA is increased during lactation involving EIF3A despite a decrease in protein synthesis machinery like RPS12 and RPS21 in the ribosomes. This results in an overall increase in milk protein synthesis and mammary gland milk protein secretion [36,37]. Another study found a decrease in ribosomal protein expression in spite of the increase in milk protein synthesis during lactation [36]. This finding suggests that the decrease or no increase in the number of ribosomal components despite the increase in beta-casein expression and milk protein secretion is a way in which the mammary gland can focus on mRNA

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Table 6. Differentially expressed proteins upon supplementation with D-Met and HMBi in MAC-T cells compared to control

Detection protein	D-Met	HMBi
Increasing number	46	40
Decreasing number	68	78
Selected downregulated and upregulated proteins		
Eukaryotic translation initiation factor 3 subunit A (EIF3A)		▲
Ribosomal protein S21 (RPS21)		▼
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	▲	
Malate dehydrogenase, mitochondrial (MDH2)	▲	▲
Eukaryotic translation initiation factor 4A1 (EIF4A1)	▼	
Eukaryotic translation initiation factor 4A2 (EIF4A2)	▲	
ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1 (ATP5A1)		
Ribosomal protein S12 (RPS12)		▼

▲, Upregulated (> 2-fold protein expression than in control); ▼, Downregulated (< 0.5-fold protein expression than in control).
D-Met, D-methionine; HMBi, 2-hydroxy-4-methylthiobutanoic acid I; MAC-T, immortalized bovine mammary epithelial cell line.

translation of proteins related to milk synthesis rather than ribosomes. This allows mammary epithelial cells to synthesize milk protein at an optimal level [36]. The ribosomes in the mammary gland decrease during lactation to favor beta-casein synthesis [36].

Pathways linked to protein and energy metabolism significantly affected by the supplemental treatments are shown in Table 7. The glycolysis pathway was activated by D-Met and HMBi treatments. The pentose phosphate pathway was activated by HMBi supplementation, and pyruvate metabolism was stimulated by D-Met supplementation. The activation by D-Met supplementation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), pyruvate metabolism, glycolysis, and

Table 7. Protein and energy metabolism-related pathways affected by supplementation treatments compared with control

Detected pathway	D-Met	HMBi
Apoptosis signaling		●
ATP synthesis		
Fructose galactose metabolism	●	
FAS signaling	●	●
Glycolysis	●	●
Heterotrimeric G-protein signaling pathway (Gi alpha- and Gs alpha-mediated)		●
Heterotrimeric G-protein signaling pathway (Gq alpha- and Go alpha-mediated)		●
Pentose phosphate		●
PI3 kinase	●	
Pyruvate metabolism	●	
Ubiquitin proteasome		●
Insulin/IGF pathway-mitogen activated protein kinase/MAP kinase cascade	●	
Insulin/IGF pathway -protein kinase B signaling cascade	●	

●, Significantly increased protein and energy metabolism-related pathways ($p < 0.05$) relative to control, run in PANTHER program for *Bos taurus* (see methods for detailed explanation).
D-Met, D-methionine; HMBi, 2-hydroxy-4-methylthiobutanoic acid i; ATP, adenosine triphosphate; PI3, phosphoinositide 3; IGF, insulin-like growth factor; MAP, mitogen-activated protein.

Table 8. List of all upregulated proteins in MAC-T cell supplemented with 0.6 mM D-Met and HMBi

	Protein ID	Protein name	Score (Protein probability > 95%)			%emPAI (Semi quantification)		
			Con	D-Met	HMBi	Con	D-Met	HMBi
1	IPI00700035	HSPA1A Heat shock 70 kDa protein 1B	175	191	254	0.316	0.440	0.728*
2	IPI00710719	AHNAK AHNAK nucleoprotein isoform 1	-	-	61	-	-	0.043*
3	IPI00696012	MYH9 myosin, heavy chain 9, non-muscle	-	230	74	-	0.560*	0.343*
4	IPI00716130	UBA1 Uncharacterized protein	105	161	143	0.172	0.240	0.428*
5	IPI01017790	PDIA6 protein disulfide-isomerase A6	123	97	149	0.460	1.000	1.071
6	IPI00889470	LOC617875 histone H4-like	-	-	97	-	-	3.556*
7	IPI00699333	ANXA8L1 Annexin A8	118	84	61	0.632	0.880	0.457*
8	IPI00867095	ALDOA Fructose-bisphosphate aldolase	98	116	70	0.574	1.240*	0.857
9	IPI00691199	LOC525863 Histone H4	-	80	-	-	3.280*	-
10	IPI01002058	NME1-NME2 protein-like	-	75	-	-	1.320*	-
11	IPI00699002	ANP32B Acidic leucine-rich nuclear phosphoprotein 32 family member B	-	107	36	-	0.520*	0.557*
12	IPI00708046	TUBB2B Tubulin beta-5 chain	-	76	75	-	0.640*	1.028*
13	IPI00717000	LOC617264 Uncharacterized protein	-	76	80	-	0.480*	0.514*
14	IPI00710895	PGK1 Phosphoglycerate kinase 1	86	94	43	0.230	1.080*	0.343
15	IPI00688257	HNRNPU Heterogeneous nuclear ribonucleoprotein U	-	37	32	-	0.160*	0.171*
16	IPI00903886	NME2 Nucleoside diphosphate kinase	32	-	51	0.373	-	1.157*
17	IPI00734138	HNRNPH1 Uncharacterized protein	-	21	39	-	0.280*	0.300*
18	IPI00707320	YWHAQ 14-3-3 protein theta	-	-	47	-	-	1.243*
19	IPI00715044	KRT17 Keratin, type I cytoskeletal 17	-	62	58	-	0.640*	0.686*
20	IPI00710991	PLS3 Plastin 3	51	31	46	0.144	0.440*	0.471*
21	IPI00695994	COPS3 COP9 signalosome complex subunit 3	-	45	-	-	0.320*	-
22	IPI00697851	KRT5 Keratin, type II cytoskeletal 5	33	-	27	0.172	-	0.514*
23	IPI00691963	CALR Calreticulin	56	25	39	0.230	0.320	0.686*
24	IPI01004191	FLNB filamin-B	-	38	-	-	0.040*	-
25	IPI00867349	CAST protein (Fragment)	-	19	20	-	0.160*	0.171*
26	IPI00707656	ANXA3 Annexin A3	-	19	28	-	0.400*	0.428*
27	IPI00699280	RPSA Similar to 40S ribosomal protein SA (Fragment)	-	37	-	-	0.840*	-
28	IPI00839940	PLCD1 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta-1	-	-	23	-	-	0.171*
29	IPI00698663	S100A4 Protein S100-A4	-	35	34	-	1.360*	1.457*
30	IPI00713814	GAPDH Glyceraldehyde-3-phosphate dehydrogenase	-	-	31	-	-	0.428*
31	IPI00717685	STIP1 Stress-induced-phosphoprotein 1	-	43	-	-	0.240*	-
32	IPI00712250	MDH2 Malate dehydrogenase, mitochondrial	-	23	30	-	0.440*	0.471*
33	IPI00713144	LRRC16B leucine-rich repeat-containing protein 16B	-	31	-	-	0.080*	-
34	IPI00903900	HIST3H2BB Histone H2B	-	-	31	-	-	1.114*
35	IPI00841695	CCT4 T-complex protein 1 subunit delta	29	43	37	0.172	0.520*	0.557*
36	IPI01028198	- 18 kDa protein	-	30	-	-	0.880*	-
37	IPI00692963	SEC23A Protein transport protein Sec23A	-	-	20	-	-	0.171*
38	IPI00708921	KRT18 keratin, type I cytoskeletal 18	-	-	30	-	-	0.343*
39	IPI00695841	TMSB10 Thymosin beta-10	-	31	-	-	3.680*	-
40	IPI00696435	YWHAE 14-3-3 protein epsilon	-	16	-	-	0.520*	-
41	IPI00696263	CAPNS1 Calpain small subunit 1	-	27	-	-	0.560*	-
42	IPI00692247	HSPA9 Stress-70 protein, mitochondrial	41	18	16	0.144	0.200	0.428*
43	IPI00709051	AGR2 Anterior gradient homolog 2	-	28	-	-	0.760*	-
44	IPI00714624	CCT3 T-complex protein 1 subunit gamma	-	26	-	-	0.240*	-

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Table 8. Continued

	Protein ID	Protein name	Score (Protein probability > 95%)			%emPAI (Semi quantification)		
			Con	D-Met	HMBi	Con	D-Met	HMBi
45	IPI00715091	RPS20 40S ribosomal protein S20	-	-	26	-	-	1.285*
46	IPI00709865	RPSA 40S ribosomal protein SA	-	-	24	-	-	0.471*
47	IPI00715705	IVL IVL protein	-	21	-	-	0.320*	-
48	IPI00867215	LRRC47 LRRC47 protein	-	23	-	-	0.240*	-
49	IPI00724124	OLA1;LOC100335717 Obg-like ATPase 1	-	23	-	-	0.320*	-
50	IPI00710228	MCRS1 Uncharacterized protein	-	-	23	-	-	0.300*
51	IPI00688180	FLNB Uncharacterized protein	-	-	17	-	-	0.043*
52	IPI00706431	LAD1 Ladinin 1	-	-	20	-	-	0.300*
53	IPI00703110	YWHAZ 14-3-3 protein zeta/delta	-	16	-	-	0.560*	-
54	IPI00716730	ORC5 ORC5L protein	-	22	-	-	0.280*	-
55	IPI00716013	MYL6 Isoform Smooth muscle of Myosin light polypeptide 6	-	22	-	-	0.920*	-
56	IPI00702582	ACOT12 Uncharacterized protein	-	22	-	-	0.240*	-
57	IPI01002054	INSR insulin receptor	-	22	-	-	0.080*	-
58	IPI00695895	Uncharacterized protein	-	-	22	-	-	0.257
59	IPI00712889	LAMB3 Laminin, beta 3	-	21	-	-	0.120*	-
60	IPI00912185	UGT2B11 UDP glucuronosyltransferase 2B10 isoform 1	-	21	-	-	0.240*	-
61	IPI01002579	KCNC2 potassium voltage-gated channel, Shaw-related subfamily, member 2, partial	-	21	-	-	0.320*	-
62	IPI00711396	ATP6V0A2 V-type proton ATPase 116 kDa subunit a isoform 2	-	-	21	-	-	0.171*
63	IPI00732001	SPTAN1 Uncharacterized protein	-	21	-	-	0.040*	-
64	IPI00838304	Uncharacterized protein	-	20	19	-	0.280*	0.300*
65	IPI00843042	NAP1L4 Nucleosome assembly protein 1-like 4	-	-	21	-	-	0.386*
66	IPI00717231	ACAA1 Uncharacterized protein	-	19	-	-	0.320*	-
67	IPI01003646	LOC100337336 Kelch-like protein 30-like	-	19	17	-	0.360*	0.386*
68	IPI00867250	GAL3ST1 Galactosylceramide sulfotransferase	-	-	16	-	-	0.343*
69	IPI00700826	USP34 hypothetical protein isoform 1	-	14	-	-	0.040*	-
70	IPI00826303	VPRBP HIV-1 Vpr binding protein, partial	-	14	-	-	0.080*	-

*Upregulated proteins in MAC-T cell supplemented with 0.6 mM D-Met and HMBi related to control.

MAC-T, immortalized bovine mammary epithelial cell line; D-Met, D-methionine; HMBi, 2-hydroxy-4-methylthiobutanoic acid i.

galactose metabolism which are energy production pathways will eventually inhibit adenosine monophosphate kinase (AMPK), a known inhibitor of mTOR, and this will result in promoting beta-casein gene expression and increase protein synthesis [38–41]. In addition, the activation of the above-mentioned pathways was expected because the treatment supplementation provides additional energy sources since D-Met and HMBi are known carbon skeleton sources.

HMBi also stimulated the glycolysis and pentose phosphate pathways, which are involved in metabolic pathways of energy production, and promote increased glucose 1-phosphate which is an energy metabolic intermediate product. These reactions could also influence mTOR stimulation by inhibiting AMPK that will increase beta-casein gene expression and milk protein synthesis [39,40]. The AMPK is a fuel-sensing enzyme that is present in mammalian cells [40]. Previous studies reported that energy intermediates indirectly activate protein synthesis by blocking AMPK, a known direct inhibitor of the mTOR pathway [7,39,40]. An increase in energy status can block the inhibition of AMPK to mTOR [34].

Table 9. List of all downregulated proteins in MAC-T cell supplemented with 0.6 mM D-Met and HMBi

	Protein ID	Protein name	Score (Protein probability > 95%)			%emPAI (Semi quantification)		
			Con	D-Met	HMBi	Con	D-Met	HMBi
1	IPI01001130	MYH9 myosin, heavy chain 9, non-muscle	181	-	-	0.488	-*	-*
2	IPI00699981	LOC617745 histone cluster 1, H2bd-like	133	74	-	0.517	0.720	-*
3	IPI00697107	TUBB2A Tubulin beta-2B chain	102	-	-	0.460	-*	-*
4	IPI01003935	LOC781223 histone cluster 1, H4j-like	93	-	-	0.833	-*	-*
5	IPI00700324	HNRNPUL2 Uncharacterized protein	86	-	-	0.115	-*	-*
6	IPI01000167	AHNAK AHNAK nucleoprotein	74	89	-	0.029	0.040	-*
7	IPI00689857	PRDX6 Peroxiredoxin-6	141	91	34	2.958	1.320*	0.643*
8	IPI00883375	MTAP;PRDX3 PRDX3 protein	60	-	-	0.345	-*	-*
9	IPI00710783	HBA1;HBA Hemoglobin subunit alpha	81	-	-	0.747	-*	-*
10	IPI00688839	NENF Neudesin	61	-	-	0.603	-*	-*
11	IPI00686173	GSTP1 Glutathione S-transferase P	83	38	39	1.637	0.640*	1.500
12	IPI00706942	TPI1 Triosephosphate isomerase	86	62	-	2.010	-*	-*
13	IPI00711327	CDH1 CDH1 protein	51	-	-	0.115	-*	-*
14	IPI00903886	NME2 Nucleoside diphosphate kinase	32	-	51	0.373	-*	1.157
15	IPI00718337	STMN1 Stathmin	53	-	-	0.632	-*	-*
16	IPI00685112	USP5 ubiquitin specific peptidase 5 (isopeptidase T) isoform 2	85	73	-	0.115	0.160	-*
17	IPI00694304	CLU Clusterin	49	-	-	0.201	-*	-*
18	IPI00968674	HMGCS1 hydroxymethylglutaryl-CoA synthase, cytoplasmic	41	-	-	0.172	-	-
19	IPI00695201	HNRNPA2B1 Heterogeneous nuclear ribonucleoproteins A2/B1	41	-	-	0.287	-*	-*
20	IPI00701642	PGD 6-phosphogluconate dehydrogenase, decarboxylating	49	39	-	0.431	0.280	-*
21	IPI00712785	COPB1 Coatomer subunit beta	44	-	-	0.086	-*	-*
22	IPI00727752	PLCD1 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta-1-like	42	-	-	0.115	-*	-*
23	IPI00697851	KRT5 Keratin, type II cytoskeletal 5	33	-	27	0.172	-*	0.514
24	IPI00715690	CTNNA1 Catenin alpha-1	32	30	-	0.230	0.160	-*
25	IPI00689750	LMNA Uncharacterized protein	44	-	-	0.144	-*	-*
26	IPI00699723	RANBP6 Uncharacterized protein	27	-	63	0.230	-*	0.343
27	IPI00715354	SFN 14-3-3 protein sigma	72	-	-	2.556	-*	-*
28	IPI00690789	MVD Diphosphomevalonate decarboxylase	37	-	-	0.258	-*	-*
29	IPI00698874	DCPS Scavenger mRNA-decapping enzyme DcpS	37	-	-	0.287	-*	-*
30	IPI00685732	EEF1B2 Elongation factor 1-beta	37	-	-	0.431	-*	-*
31	IPI00718050	DYNLRB1 Dynein light chain roadblock-type 1	37	-	-	1.034	-*	-*
32	IPI00713760	GDI2 Rab GDP dissociation inhibitor beta	59	29	-	0.201	0.280	-*
33	IPI00710020	S100A14 Protein S100-A14	36	-	-	1.005	-*	-*
34	IPI01003068	LOC100337475 ribosomal protein SA-like	23	-	-	0.603	-*	-*
35	IPI00685123	TLN1 talin-1	41	47	-	0.029	0.040	-*
36	IPI00692093	ANXA5 Annexin A5	53	-	36	0.287	-*	0.428
37	IPI01001213	TCHH trichohyalin isoform 1	37	33	-	0.057	0.080	-*
38	IPI00709590	KRT4 KRT4 protein	35	-	-	0.172	-*	-*
39	IPI00760419	RPS21 40S ribosomal protein S21	28	41	-	1.292	1.800	-*
40	IPI00845184	KRT6A KRT6A protein	33	-	38	0.172	-*	0.257
41	IPI00694938	SERPINH1 Serpin H1	39	-	-	0.230	-*	-*
42	IPI00695508	CALM3;CALM Calmodulin	32	-	29	0.661	-*	0.985
43	IPI00715508	PAFAH1B3 Platelet-activating factor acetylhydrolase IB subunit gamma	31	-	-	0.431	-*	-*
44	IPI00691137	LOC100137883;LOC100297716;TMSB4 Thymosin beta-4	31	-	-	0.7237	-*	-*
45	IPI00686092	PRDX1 Peroxiredoxin-1	48	15	-	0.488	0.680	-*
46	IPI00705354	ATOX1 Copper transport protein ATOX1	24	-	17	1.637	-*	2.442
47	IPI00702790	MACF1 microtubule-actin cross-linking factor 1	30	-	-	0.029	-*	-*

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Table 9. Continued

	Protein ID	Protein name	Score (Protein probability > 95%)			%emPAI (Semi quantification)		
			Con	D-Met	HMBi	Con	D-Met	HMBi
48	IPI00686546	CCT2 T-complex protein 1 subunit beta	31	58	-	0.172	1.240	-*
49	IPI00718444	CTNND1 catenin delta-1	28	19	-	0.086	0.120	-*
50	IPI00687409	LOC100336093;MYL12B Myosin regulatory light chain 12B	37	-	-	0.574	-*	-*
51	IPI00699092	TTC39B tetratricopeptide repeat domain 39B	29	-	-	0.144	-*	-*
52	IPI00693929	DCTN2 Dynactin subunit 2	24	21	-	0.230	0.320	-*
53	IPI00694142	MIF Macrophage migration inhibitory factor	31	26	-	0.919	1.280	-*
54	IPI00710727	VCP Transitional endoplasmic reticulum ATPase	39	-	28	0.230	-*	0.171
55	IPI00712042	RNH1 Ribonuclease/angiogenin inhibitor 1	37	19	-	0.230	0.320	-*
56	IPI00707751	EEF2 Elongation factor 2	47	-	28	0.115	-*	0.171
57	IPI00712133	FASN Fatty acid synthase	36	27	19	0.086	0.120	0.043*
58	IPI00706632	EEF1G Elongation factor 1-gamma	33	-	23	0.431	-*	0.300
59	IPI00906952	Uncharacterized protein	33	-	-	0.316	-*	-*
60	IPI00695419	DBI Acyl-CoA-binding protein	36	-	-	1.149	-*	-*
61	IPI00924223	ZEB2 Uncharacterized protein	27	-	-	0.086	-*	-*
62	IPI01017521	ASPM Abnormal spindle-like microcephaly-associated protein homolog (Fragment)	27	-	-	0.029	-*	-*
63	IPI00709465	P4HB Protein disulfide-isomerase	27	-	-	0.201	-*	-*
64	IPI00688816	CLTC Clathrin heavy chain 1	36	28	-	0.057	0.080	-*
65	IPI00691663	PLS1 Plastin-1	40	22	-	0.144	0.200	-*
66	IPI00716748	BCAM Basal cell adhesion molecule	33	19	-	0.144	0.200	-*
67	IPI00713901	EIF2S2 Eukaryotic translation initiation factor 2 subunit 2	29	23	-	29	23	-*
68	IPI00704353	TUBA4A Tubulin alpha-4A chain	29	23	-	0.201	0.280	-*
69	IPI00711750	ARPC3 Actin-related protein 2/3 complex subunit 3	32	-	-	0.546	-*	-*
70	IPI01001324	LOC533883 KIAA1671 protein-like	25	-	-	0.057	-*	-*
71	IPI00714708	DHX9 ATP-dependent RNA helicase A	25	-	-	0.086	-*	-*
72	IPI00903876	Uncharacterized protein	25	-	-	0.747	-*	-*
73	IPI00701894	JUP Junction plakoglobin	35	14	-	0.144	0.200	-*
74	IPI00711338	LONP2 Lon protease homolog 2, peroxisomal	24	-	-	0.115	-*	-*
75	IPI00698179	HSPA4 Uncharacterized protein	17	-	-	0.115	-*	-*
76	IPI00704325	WARS Tryptophanyl-tRNA synthetase, cytoplasmic	23	-	-	0.201	-*	-*
77	IPI00690680	HDDC3 HD domain-containing protein 3	23	-	-	0.546	-*	-*
78	IPI00727417	MSN Moesin	29	16	-	0.144	0.200	-*
79	IPI01002905	HSPD1 60 kDa heat shock protein, mitochondrial	30	23	-	0.546	0.240*	-*
80	IPI00713536	RPS12 40S ribosomal protein S12	22	25	-	0.775	1.080	-*
81	IPI00685613	LOC100295775 Uncharacterized protein	20	21	-	0.976	1.360	-*
82	IPI01004262	LOC100337442 ribosomal protein L35-like	29	-	13	0.172	-*	0.257
83	IPI01028151	STAT1 134 kDa protein	24	-	24	0.086	-*	0.129
84	IPI00715345	PC Pyruvate carboxylase, mitochondrial	16	-	32	0.086	-*	0.129
85	IPI00696935	HNRNPD Uncharacterized protein	19	22	-	0.287	0.400	-*
86	IPI01003143	SHROOM2 SHROOM2-like, partial	21	-	-	0.057	-*	-*
87	IPI00709517	DIAPH1 diaphanous homolog 1-like	24	-	-	0.086	-*	-*
88	IPI00700509	CCT7 T-complex protein 1 subunit eta	24	-	20	0.172	-*	0.257
89	IPI00716534	DDX1 DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	20	-	-	0.115	-*	-*
90	IPI00685601	RPS3 40S ribosomal protein S3	20	-	-	0.402	-*	-*
91	IPI00710247	KPNB1 Uncharacterized protein	15	-	-	0.115	-*	-*
92	IPI00698249	MYH10 Nonmuscle myosin heavy chain B (Fragment)	20	-	-	1.149	-*	-*
93	IPI00689902	NPM1 Nucleophosmin	16	-	16	0.345	-*	0.514
94	IPI00692105	SELV selenoprotein V	14	-	13	0.258	-*	0.386

*Downregulated proteins in MAC-T cell supplemented with 0.6 mM D-Met and HMBi related to control.

MAC-T, immortalized bovine mammary epithelial cell line; D-Met, D-methionine; HMBi, 2-hydroxy-4-methylthiobutanoic acid i.

In the analysis of intracellular AA levels inside MAC-T cells, intracellular Met levels in MAC-T cells increased significantly in the D-Met supplemented cells (Fig. 5; $p < 0.05$). The lack of increase in cell-free Met in HMBi treatment suggests that a small amount of HMBi was converted to Met within the cells. Thus a direct reaction of HMBi with the cell could be possible, as shown in the significantly higher beta-casein and S6K1 gene expression and numerically higher medium protein quantity in HMBi compared to D-Met treatment and control groups.

This trend is also seen in serine, glumatic acid, and aspartic acid. There was also a higher concentration of isoleucine found inside the cells in the D-Met and HMBi groups compared to control ($p < 0.05$). The D-Met group has a higher intracellular concentration of leucine and tyrosine than the control but is not significantly different from the HMBi group ($p < 0.05$).

Metabolome analysis

Pathway metabolite detection results are listed in Table 10. Most pathways related to the detected metabolites were involved in energy and AA metabolism. Energy pathways such as glycolysis, gluconeogenesis, and the pyruvate metabolism pathway were activated by D-Met supplementation. The detected metabolites affected by supplemental treatments are listed in Table 11. D-Met supplementation increased the production of seven metabolites and decreased UMP. HMBi supplementation increased the production of three metabolites (and decreased that of UMP and N-acetyl-L-glutamate). It also increased the production of the energy metabolite glucose 1-phosphate.

To illustrate the interactions of different Met treatments in MAC-T cells, diagrams of the relationships among various components were created (Figs. 6 and 7). Fig. 6 shows the increased relative gene expression of beta-casein, S6K1 and EIF4A2, upregulation of GADPH protein and the stimulation of glycolysis, galactose and pyruvate metabolism that resulted in to increase in milk protein synthesis upon addition of D-Met. Fig. 7 shows that upon the addition of HMBi, there were increases in the relative gene expression of beta-casein, S6K1, and EIF3A, stimulation of glycolysis and pentose phosphate pathways, and increased in the glucose 1-phosphate metabolite.

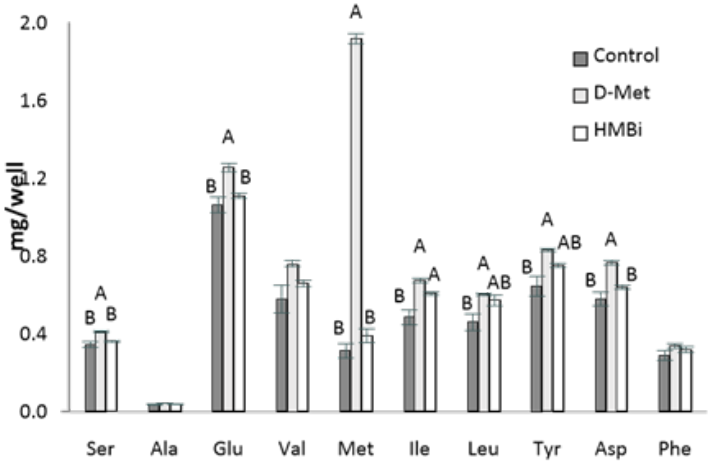

Fig. 5. The amino acid content in MAC-T cells was supplemented with control, D-Met (+0.6 mM), and HMBi (+0.6 mM). The values are means ± SE (n = 3). A, B, indicate significant differences at $p < 0.05$ by least significant difference (LSD) test. MAC-T, immortalized bovine mammary epithelial cell line; D-Met, D-methionine; HMBi, 2-hydroxy-4-methylthiobutanoic acid i.

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Table 10. Metabolic pathways affected by supplementation treatment compared with control

Metabolite-related pathway	D-Met	HMBi
Alanine, aspartate, and glutamate metabolism	●	
Aminoacyl-tRNA biosynthesis	●	●
Arginine and proline metabolism	●	
β-Alanine metabolism	●	
Butanoate metabolism		
Citrate cycle (TCA cycle)		
Cysteine and methionine metabolism	●	
D-Glutamine and D-glutamate metabolism	●	
Glycine, serine, and threonine metabolism	●	
Glycolysis or gluconeogenesis	●	
Glyoxylate and dicarboxylate metabolism		
Histidine metabolism		
Inositol phosphate metabolism	●	
Pantothenate and CoA biosynthesis		
Pentose phosphate pathway	●	
Phenylalanine metabolism	●	
Propanoate metabolism		
Pyruvate metabolism	●	
Tyrosine metabolism	●	
Ubiquinone and another terpenoid-quinone biosynthesis		

●, Significantly increased pathway metabolites determined by Metabo Analyst v. 3.0 software for *Bos taurus*, relative to control ($p < 0.05$) (see Methods for detailed explanation).

●, Pathway detection metabolites affected by the application of treatments were further analyzed by Dunnett's Multiple Comparison test, a multiple comparison procedure to compare each of several treatments with a single control.

D-Met, D-methionine; HMBi, 2-hydroxy-4-methylthiobutanoic acid i.

Table 11. Metabolites affected by supplementation treatments compared with control

The observed change in the production	D-Met	HMBi
Increase	Serine	Glucose 1-phosphate
	Glutamine	6-Phosphogluconate
	Methionine	Isoleucine
	Aspartate	
	Isoleucine	
	Leucine	
	Tyrosine	
Decrease	Uridine monophosphate	Uridine monophosphate
		N-Acetyl-L-glutamate

Metabolite analysis (*Bos taurus*). Increased and decreased metabolites were further analyzed by Dunnett's Multiple Comparison test which is a multiple comparison procedure for treatments with a single control.

D-Met, D-methionine; HMBi, 2-hydroxy-4-methylthiobutanoic acid i.

CONCLUSION

Taken together, these results show that D-Met and HMBi are effective for stimulating milk protein synthesis. D-Met and HMBi supplementation effectively stimulated beta-casein and S6K1 mRNA expression. They are also effective and efficient in activating pathways linked with milk protein

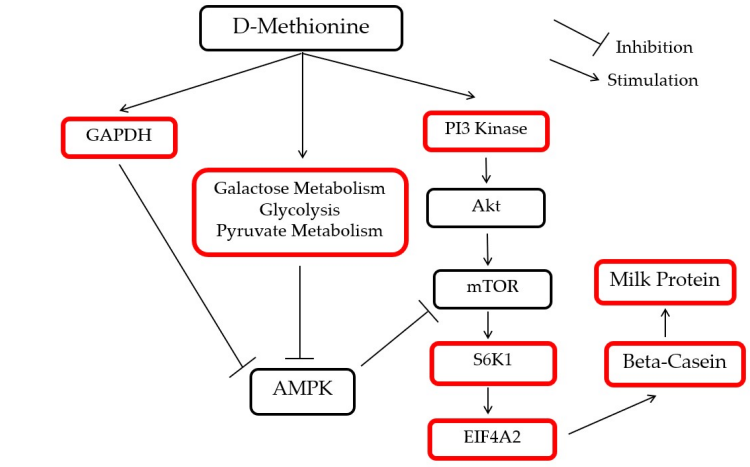

Fig. 6. Diagram of the effect of D-Met supplementation on the milk protein synthesis pathway. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PI3 kinases, phosphoinositide 3-kinases; mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase; S6K1, S6 kinase beta-1; D-Met, D-methionine.

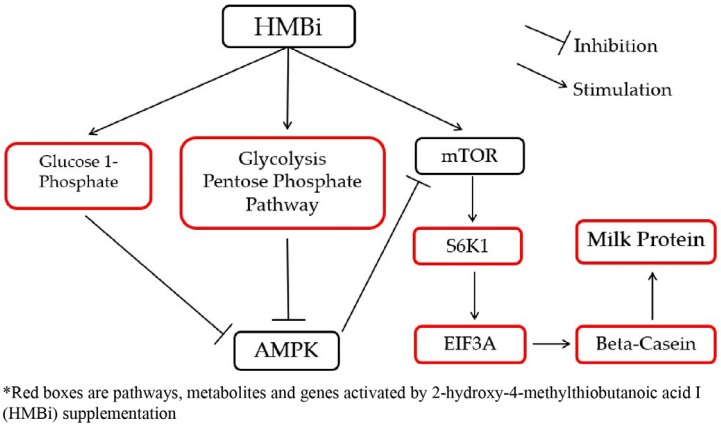

Fig. 7. Diagram of the effect of HMBi addition on the beta-casein synthesis pathway. HMBi, 2-hydroxy-4-methylthiobutanoic acid i; mTOR, mammalian target of rapamycin; S6K1, S6 kinase beta-1; AMPK, adenosine monophosphate kinase; EIF3A: eukaryotic translation initiation factor 3 subunit.

synthesis and increasing the expression of proteins and the production of metabolites involved in milk protein synthesis.

REFERENCES

1. NRC [National Research Council]. Subcommittee on dairy cattle nutrition. Committee on animal nutrition, board on agriculture and natural resources. In: Nutrient requirements of dairy cattle. 7th rev. ed. Washington, DC: The National Academies Press; 2001.
2. Métayer S, Seiliez I, Collin A, Duchêne S, Mercier Y, Geraert PA, et al. Mechanisms through which sulfur amino acids control protein metabolism and oxidative status. J Nutr Biochem. 2008;19:207-15. https://doi.org/10.1016/j.jnutbio.2007.05.006
3. Orellana RA, Jeyapalan A, Escobar J, Frank JW, Nguyen HV, Suryawan A, et al. Amino acids augment muscle protein synthesis in neonatal pigs during acute endotoxemia by stimulating

https://doi.org/10.5187/jast.2022.e37

mTOR-dependent translation initiation. Am J Physiol Endocrinol Metab. 2007;293:E1416-25. https://doi.org/10.1152/ajpendo.00146.2007
4. Kimball SR, Jefferson LS. Amino acids as regulators of gene expression. Nutr Metab. 2004;1:3. https://doi.org/10.1186/1743-7075-1-3
5. Huang YL, Zhao F, Luo CC, Zhang X, Si Y, Sun Z, et al. SOCS3-mediated blockade reveals major contribution of JAK2/STAT5 signaling pathway to lactation and proliferation of dairy cow mammary epithelial cells in vitro. Molecules. 2013;18:12987-3002. https://doi.org/10.3390/molecules181012987
6. Zhang X, Zhao F, Si Y, Huang Y, Yu C, Luo C, et al. GSK3β regulates milk synthesis in and proliferation of dairy cow mammary epithelial cells via the mTOR/S6K1 signaling pathway. Molecules. 2014;19:9435-52. https://doi.org/10.3390/molecules19079435
7. Ranga Niroshan Appuhamy JAD, Knoebel NA, Deepthi Nayananjalie WA, Escobar J, Hanigan MD. Isoleucine and leucine independently regulate mTOR signaling and protein synthesis in MAC-T cells and bovine mammary tissue slices. J Nutr. 2012;142:484-91. https://doi.org/10.3945/jn.111.152595
8. Toerien CA, Trout DR, Cant JP. Nutritional stimulation of milk protein yield of cows is associated with changes in phosphorylation of mammary eukaryotic initiation factor 2 and ribosomal s6 kinase 1. J Nutr. 2010;140:285-92. https://doi.org/10.3945/jn.109.114033
9. Deval C, Chaveroux C, Maurin AC, Cherasse Y, Parry L, Carraro V, et al. Amino acid limitation regulates the expression of genes involved in several specific biological processes through GCN2-dependent and GCN2-independent pathways. FEBS J. 2009;276:707-18. https://doi.org/10.1111/j.1742-4658.2008.06818.x
10. Jousse C, Averous J, Bruhat A, Carraro V, Mordier S, Fafournoux P. Amino acids as regulators of gene expression: molecular mechanisms. Biochem Biophys Res Commun. 2004;313:447-52. https://doi.org/10.1016/j.bbrc.2003.07.020
11. Dibner JJ, Knight CD. Conversion of 2-hydroxy-4-(methylthio)butanoic acid to L-methionine in the chick: a stereospecific pathway. J Nutr. 1984;114:1716-23. https://doi.org/10.1093/jn/114.9.1716
12. Saunderson CL. Comparative metabolism of L-methionine, DL-methionine and DL-2-hydroxy 4-methylthiobutanoic acid by broiler chicks. Br J Nutr. 1985;54:621-33. https://doi.org/10.1079/bjn19850149
13. Dupuis L, Saunderson CL, Puigserver A, Brachet P. Oxidation of methionine and 2-hydroxy 4-methylthiobutanoic acid stereoisomers in chicken tissues. Br J Nutr. 1989;62:63-75. https://doi.org/10.1079/bjn19890008
14. Polan CE, Chandler PT, Miller CN. Methionine hydroxy analog: varying levels for lactating cows. J Dairy Sci. 1970;53:607-10. https://doi.org/10.3168/jds.s0022-0302(70)86264-6
15. Patterson JA, Kung L Jr. Metabolism of DL-methionine and methionine analogs by rumen microorganisms. J Dairy Sci. 1988;71:3292-301. https://doi.org/10.3168/jds.s0022-0302(88)79934-8
16. Robert J, Ferragu V, Bouza B, Williams PEV. Influence of different sources of methionine (DL methionine, hydroxyl-analogues of methionine) and sulphur on the digestibility of organic matter of maize silage measured in vitro. In: Proceedings of the British Society of Animal Science. Scarborough: Cambridge University Press; 1998.
17. Sloan BK, Hoover WH, Miller Webster TK, Schwab CG, Whitehouse NL. Action of hydroxy methyl butanoic acid (HMB) on microbial growth and metabolism. J Dairy Sci. 2000:83:269
18. Dong X, Zhou Z, Saremi B, Helmbrecht A, Wang Z, Loor JJ. Varying the ratio of Lys:Met while maintaining the ratios of Thr:Phe, Lys:Thr, Lys:His, and Lys:Val alters mammary cellular

metabolites, mammalian target of rapamycin signaling, and gene transcription. J Dairy Sci. 2018;101:1708-18. https://doi.org/10.3168/jds.2017-13351
19. Conejos JRV, Nejad JG, Kim JE, Moon JO, Lee JS, Lee HG. Supplementing with l-tryptophan increases medium protein and alters expression of genes and proteins involved in milk protein synthesis and energy metabolism in bovine mammary cells. Int J Mol Sci. 2021;22:2751. https://doi.org/10.3390/ijms22052751
20. Kim J, Lee HG. Effects of L-histidine and sodium acetate on β-casein expression in nutrient-restricted bovine mammary epithelial cells. Animals. 2021;11:1444. https://doi.org/10.3390/ani11051444
21. Wang T, Lim JN, Bok JD, Kim JH, Kang SK, Lee SB et al. Association of protein expression in isolated milk epithelial cells and cis-9, trans-11 conjugated linoleic acid proportions in milk from dairy cows. J Sci Food Agric. 2014;94:1835-43. https://doi.org/10.1002/jsfa.6502
22. Wang T, Lee SB, Hwang JH, Lim JN, Jung US, Kim MJ, et al. Proteomic analysis reveals PGAM1 altering cis-9, trans-11 conjugated linoleic acid synthesis in bovine mammary gland. Lipids. 2015;50:469-81. https://doi.org/10.1007/s11745-015-4009-9
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT Method. Methods. 2001;25:402-8. https://doi.org/10.1006/meth.2001.1262
24. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, et al. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics. 2005;4:1265-72. https://doi.org/10.1074/mcp.m500061-mcp200
25. Park C, Yun S, Lee SY, Park K, Lee J. Metabolic profiling of Klebsiella oxytoca: evaluation of methods for extraction of intracellular metabolites using UPLC/Q-TOF-MS. Appl Biochem Biotechnol. 2012;167:425-38. https://doi.org/10.1007/s12010-012-9685-9
26. Kim YE, Jeon HJ, Kim D, Lee SY, Kim KY, Hong J, et al. Quantitative proteomic analysis of 2D and 3D cultured colorectal cancer cells: profiling of tankyrase inhibitor XAV939-induced proteome. Sci Rep. 2018;8:13255. https://doi.org/10.1038/s41598-018-31564-6
27. Lu L, Gao X, Li Q, Huang J, Liu R, Li H. Comparative phosphoproteomics analysis of the effects of L-methionine on dairy cow mammary epithelial cells. Can J Anim Sci. 2012;92:433-42. https://doi.org/10.4141/cjas2012-063
28. Jeon SW, Conejos JR, Kim J, Kim MJ, Lee JE, Lee BS, et al. Supplementing conjugated and non-conjugated L-methionine and acetate alters expression patterns of CSN2, proteins and metabolites related to protein synthesis in bovine mammary cells. J Dairy Res. 2020;87:70-7. https://doi.org/10.1017/s0022029919000979
29. Nan X, Bu D, Li X, Wang J, Wei H, Hu H, et al. Ratio of lysine to methionine alters expression of genes involved in milk protein transcription and translation and mTOR phosphorylation in bovine mammary cells. Physiol Genomics. 2014;46:268-75. https://doi.org/10.1152/physiolgenomics.00119.2013
30. Reynolds CK, Harmon DL, Cecava MJ. Absorption and delivery of nutrients for milk protein synthesis by portal-drained viscera. J Dairy Sci. 1994;77:2787-808. https://doi.org/10.3168/jds.s0022-0302(94)77220-9
31. Shennan DB, Peaker M. Transport of milk constituents by the mammary gland. Physiol Rev. 2000;80:925-51. https://doi.org/10.1152/physrev.2000.80.3.925
32. Cant JP, Kim JJM, Cieslar SRL, Doelman J. Symposium review: amino acid uptake by the mammary glands: Where does the control lie? J Dairy Sci. 2018;101:5655-66. https://doi.org/10.3168/jds.2017-13844

https://doi.org/10.5187/jast.2022.e37

33. Kim J, Lee JE, Lee JS, Park JS, Moon JO, Lee HG. Phenylalanine and valine differentially stimulate milk protein synthetic and energy-mediated pathway in immortalized bovine mammary epithelial cells. J Anim Sci Technol. 2020;62:263-75. https://doi.org/10.5187/jast.2020.62.2.263
34. Proud CG. Signalling to translation: how signal transduction pathways control the protein synthetic machinery. Biochem J. 2007;403:217-34. https://doi.org/10.1042/bj20070024
35. O'Donohue MF, Choesmel V, Faubladier M, Fichant G, Gleizes PE. Functional dichotomy of ribosomal proteins during the synthesis of mammalian 40S ribosomal subunits. J Cell Biol. 2010;190:853-66. https://doi.org/10.1083/jcb.201005117
36. Bionaz M, Periasamy K, Rodriguez-Zas SL, Everts RE, Lewin HA, Hurley WL, et al. Old and new stories: revelations from functional analysis of the bovine mammary transcriptome during the lactation cycle. PLOS ONE. 2012;7:e33268. https://doi.org/10.1371/journal.pone.0033268
37. Mercier JC, Gaye P. Milk protein synthesis. In: Mepham, TB, editor. Biochem lactation. Amsterdam: Elsevier Science; 1983. p. 177-227.
38. Berg JM, Tymoczko JL, Stryer L. Glycolysis is an energy-conversion pathway in many organisms. In: Biochemistry. 5th ed. New York: W.H. Freeman; 2002.
39. Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr Opin Cell Biol. 2005;17:596-603. https://doi.org/10.1016/j.ceb.2005.09.009
40. Richter EA, Ruderman NB. AMPK and the biochemistry of exercise: implications for human health and disease. Biochem J. 2009;418:261-75. https://doi.org/10.1042/bj20082055
41. Appuhamy JADRN, Nayananjalie WA, England EM, Gerrard DE, Akers RM, Hanigan MD. 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. J Dairy Sci. 2014;97:419-29. https://doi.org/10.3168/jds.2013-7189