Supplementing D-type and L-type Methionine and its precursor alters the expression of mRNA, proteins and metabolites involved in milk protein synthesis and energy metabolism in bovine mammary cells

S. W. Jeon[*,#], J. R. V. Conejos[*,†,#], J. E. Kim[*,†], M. J. Kim[*], J. E. Lee ‡, B. S. Lee ‡, J. S. Park ‡, J. O. Moon ‡, J.S. Lee[*,†] and H. G. Lee[*,†,1]

[*] *Department of Animal Science and Technology, Konkuk University, Seoul, Republic of Korea*

[†] *Team of Educational Program for Specialists in Global Animal Science, Brain Korea 21 Plus Project, Konkuk University, Seoul, Republic of Korea*

[‡] *CJ CheilJedang Research Institute of Biotechnology, Suwon, Republic of Korea*

[#] *Equal Contribution Status*

This study was conducted to investigate the effect of supplementing different types of methionine (L and D-type) and its precursor (HMBi) on milk protein synthesis using immortalized bovine mammary epithelial cell line (MAC-T Cell); D-methionine (D-Met), L-methionine (L-met) and 2-hydroxy-4-methylthiobutanoic acid I (HMBi), an isopropyl ester of the hydroxy analogue precursor of methionine. The underlying mechanism of milk protein synthesis by adding D- and L-type amino acid as well as HMBi was elucidated through omics analysis to verify the metabolism pathway. Results showed that HMBi group showed the highest beta casein mRNA expression levels compared to D- and L-Met groups and highest medium protein although not different with the L-Met treatment. The observed upregulated (>2 protein expression vs. control) and downregulated (<0.5 protein expression vs. control) proteins in L-Met, D-Met and HMBi treated groups were: 39, 77; 46, 68; and 40, 78, respectively. Interestingly, based on protein pathway analysis, L-Met treated group stimulated the ATP synthesis, PI3 kinase and pyruvate metabolism. On the other hand, the D-Met group stimulated fructose-galactose metabolism, glycolysis pathway, PI3 kinase and pyruvate

[1] Corresponding author: hglee66@konkuk.ac.kr,

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metabolism. And lastly HMBi-treated group stimulated pentose phosphate pathway and glycolysis pathway. Metabolite analysis revealed that L-Met treated group resulted in the increase in 11 metabolites. On the other hand, D-Met treated group showed increase in 7 metabolites and decreased of uridine monophosphate (UMP). HMBi supplementation caused increases of 3 metabolites and decreased of UMP and N-acetyl-L-glutamate Addition of different isoforms of Met stimulated the production of intermediate metabolites for energy production. Addition of L-Met stimulated the production of energy metabolites such as pyruvate, malate, and fumarate, well-known as intermediates of Krebs cycle. On the other hand, HMBi supplementation resulted in increases of energy metabolite glucose 1-phosphate and 6-phosphogluconate. Results showed that HMBi-treated group exhibited highest expression of *β-casein* mRNA expression by stimulating proteins and metabolites as well as protein and metabolic pathways involved in protein and energy synthesis. As a result, HMBi-treated group resulted in highest protein concentration but not significantly different with L-Met. Both the D- and L-isoforms has considerably the same medium protein concentration and β-casein mRNA expression higher than the control. So, D- and L-Met isoforms can be used alternatively without any significant change in protein synthesis efficiency in bovine mammary epithelial cells.

Key Words: Methionine, milk protein synthesis, proteome, HMBi

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INTRODUCTION

Methionine (Met) has been considered as the most limiting amino acid (AA) for milk protein synthesis in lactating dairy cows (National Research Council, 2001). Methionine basically exists in two basic forms, as D-methionine (D-Met) and L-methionine (L-Met) forms. Although the chemical composition of these two methionine isomers is considerably the same, their configuration is very different. The main difference between D and L forms of methionine is found in their three-dimensional structure around the central carbon. It is important to consider that enzymes are so specific to the configurations of these isomers and the animals can only convert the L-methionine into protein inside the body.

Met and its hydroxy analogue precursor like other amino acids when given to ruminants are readily degraded by the ruminal microflora (Patterson and Kung, 1988). Data from Volden et al. (1998) indicate only a small amount (about 20 %) of Met can escape rumen fermentation so that ruminal protection is very necessary.

The Met hydroxy analog, 2-hydroxy-4-(methylthio) butanoacid acid (HMB), has long been proposed as means in providing Met to increase milk and protein yields of dairy cows fed diets limited in Met (Polan et al., 1970). The Met hydroxy analogue (HMB) serves as a precursor of Met when fed to animals. Its chemical structure is basically similar to that of Met containing the carboxyl group, hydrogen atom and R group attached to the central carbon atom. The main difference is that instead of an amine group, the HMB has hydroxyl group (OH) instead. The analogue could also exist in both the D and L-isomer forms. Commercially available Met and Met hydroxy analogue being produced by chemical methods produce both the D- and L-Met equally. But because of possible manipulation and improved manufacturing process, commercial Met produced by fermentation results only in L-isomer form. HMB has a lot of beneficial effects on rumen fermentation which includes enhancement of cellulolytic activity, fiber degradation and increased acetate production (Patterson and Kung, 1988, Robert *et al*., 1998; Sloan *et al*., 2000.). Unfortunately it is extensively degraded and rapidly utilized

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by the rumen microflora. The isopropyl derivative of HMB (HMBi) has the same function as HMB but there is an additional ester group to provide additional protection against microbial degradation in the rumen. HMBi is the technical name given to a liquid product composed of around 90% by weight of the isopropyl ester of 2-hydroxy-4-methylthiobutanoic acid (HMB).

D- and L-Met inside animal's body are transported across the intestinal wall mainly by active and carrier-mediated active transport and sodium-dependent transporters and via energy and sodium-independent transporters (Knight et al., 1994). L-Met is directly incorporated into protein. On the other hand, D-Met is converted into L-Met via two-step process first via oxidation reaction by amino acid oxidase removing the amine group to form the intermediate 2-keto-4-methylthiobutanoic and then followed by the action of transaminase attaching to the amine group to form the L-Met (Dibner and Knight, 1984; Saunderson, 1985; Dupuis et al., 1989). However, the D- and L-isomers of Met hydroxy analogue are transported across intestinal wall mainly by diffusion (Knight and Dibner, 1984). The analogue undergoes a two-step process to convert into L-Met. The enzymes hydroxy acid dehydrogenase (for D-Met analogue) and hydroxy acid oxidase (for L-Met analogue) first convert the hydroxyl group into a keto group which forms the α-keto-Met. Finally, transaminase enzyme then attaches to amine group to form L-Met (Knight and Dibner, 1984). So, therefore, the L-Met molecules derived from D-Met or Met hydroxy analogue at last can then be used by the animal to build protein.

Met has a major role in protein synthesis as well as many other biochemical and cellular processes (Orellana et al., 2007; Metayer et al., 2008). Met first and foremost has a major role in the initiation of mRNA translation by the binding of methionyl-tRNA to the 40S ribosomal subunit to join with 60S ribosomal subunit to form the 80S ribosome (Kimball and Jefferson, 2004). Met supplementation also caused an increase in the protein expression and phosphorylation of STAT5a and mTOR and increase β-casein protein expression (Huang et al., 2013). In the study of Zhang et al. (2014) by supplementing Met in bovine mammary

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epithelial cells, there was an increased in activation of the mTOR pathway and an observed increased phosphorylation of mTOR inhibitor Glycogen Synthase Kinase 3. In support to this, another study from Appuhamy et al. (2012) showed positive effects in bovine mammary epithelial cells of Met supplementation on milk protein synthesis via phosphorylation of mTOR pathway-related proteins. In addition, infusion of Met in bovine mammary tissue increased the phosphorylation of S6K1 and RPS6 (Toerien et al., 2010). Many past studies had shown that through nutrient/gene interaction, Met can directly regulate the expression of different target milk protein synthesis related genes (Deval et al., 2009; Jousse et al., 2004). In amino acid combination study by Nan et al. (2014), they found out a strong nutrigenomic effect of combining Met and Lys to supplement in bovine mammary epithelial cells by significantly increasing expression of genes coding for major milk proteins, milk protein translation proteins (ELF5, MTOR) and JAK-STAT signaling (*JAK2*, *STAT5*).

A study was also conducted to compare the efficacy of N-Acetyl-L-methionine, a conjugated form of L-methionine with acetate, and its digested forms (L-met and acetate) on milk protein synthesis using an immortalized bovine mammary epithelial cell line (Conejos et al., 2017). In this study, the metabolite analysis revealed an increase in 12 metabolites and a decrease in uridine monophosphate (UMP) for N-Acetyl-L-methionine-treated group. For the other group, the non-conjugated L-Met + acetate–treated group, showed the increase of 13 metabolites and the decrease of inosine monophosphate (IMP) and pantothenate. On another study by applying gas chromatography–mass spectrometry (GC-MS) metabolomics study, Dong et al. (2018) reported some distinct clusters of differentially concentrated metabolites when treated with different ratios of Lys and Met. Until now, based on available data, as far as we know, there are still no studies being done regarding comparing the mechanism of protein synthesis by supplementing different types of Met through omics analysis.

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SUPPLEMENTING D-TYPE AND L-TYPE METHIONINE AND ITS PRECURSOR

The objectives of this study is to investigate the effect of adding different types of Met (D and L-type) and its precursor (HMBi) on: (1) milk protein synthesis using immortalized bovine mammary epithelial cell line (MAC-T Cell), and (2) to verify the metabolic pathway.

MATERIALS AND METHODS

Amino Acid Dosage and Sampling Time

Immortalized mammary epithelial cell line (MAC-T) (University of Vermont, Burlington, VT, USA) was seeded into 10-cm dishes (TPP, Trasadingen, Switzerland) and then cultured in DMEM/F12 basic medium (Thermo Scientific, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin/streptomycin (Thermo Scientific, South Logan, UT, USA), 5 μg/mL insulin, 1 μg/mL hydrocortisone, and 50 μg/mL gentamycin (Sigma-Aldrich Corp., St. Louis, MO, USA) at 37 ℃ in a 5% CO2 incubator (Wang et al., 2014; Wang et al., 2015). Then, the cells were seeded into BD Falcon™ 6-well multiwall plate (Franklin Lakes, NJ, USA) and incubated with basic growth medium.

When MAC-T Cells reached about 90% confluence, DMEM/F12 basic medium was replaced with lactogenic DMEM/F12 differentiation medium. Lactogenic DMEM/F12 differentiation medium (without FBS) was used to differentiate MAC-T cells to differentiate into *β-casein* secreting cells. This medium contained 5 μg/mL bovine insulin, 1 μg/mL hydrocortisone, 100 units/mL penicillin/streptomycin, 50 μg/mL gentamycin, and 5 μg/mL prolactin (Sigma-Alrich Corp., St. Louis, MO, USA) (Wang et al., 2014; Wang et al., 2015). The AA profile of differentiation medium was as follows: Arg, Cys, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, and Val at 0.70, 0.10, 2.5, 0.25, 0.15, 0.42, 0.45, 0.50, 0.12, 0.22, 0.25, 0.45, 0.04, 0.21, and 0.45 mM, respectively.

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SUPPLEMENTING D-TYPE AND L-TYPE METHIONINE AND ITS PRECURSOR

A preliminary study was conducted to determine the effects of different dosages of supplemental L-Met and its culture time on *β-casein* mRNA expression. Additional L-Met (0, 0.3, 0.6, 0.9 mM) was supplemented to lactogenic medium in the absence of FBS. Cells were harvested 24 hours after treatment. To evaluate the effect of culture time on *β-casein* secretion on MAC-T Cells, the cells were harvested at 0, 12, 24, 36, 48, 72, 96, and 120 h after incubated with the control medium (0mM). Each treatment was replicated six times. Data obtained were used to determine the best concentration and culture time for treatment supplementation of L-Met. After preliminary study, the best dosage and time determined were used uniformly for all treatments: L-Met, D-Met and HMBi (CJ CheilJedang Corp., Suwon, Republic of Korea). Each treatment was replicated six times.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from MAC-T cells using TRIzol® (Life Technologies Corporation, Carlsbad, CA, USA). RNA quality and quantity were determined using NanoDrop 1000® Spectrophotometer with RNA-40 module (Thermo Fisher Scientific, Wilmington, DE, USA). Then cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. After incubation at 25 ℃ for 5 min, 42 ℃ for 30 min, and 85 ℃ for 5 min, the cDNA was quantified using ssDNA-33 module of Thermo NanoDrop 1000® Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Real-Time PCR

Real-Time Polymerase Chain Reaction (RT-PCR) was performed with a total volume of 20 μL per well in 96-well microwell plates (Sigma-Alrich Corp., St. Louis, MO, USA) and a T100™ Thermal Cycler System (Bio-Rad Laboratories, Hercules, CA, USA). *β-actin* was used as a reference gene. The reaction mixture contained 50 ng cDNA, 0.6 μL forward primer,

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0.6 µL reverse primer, 10 µL Sybr Master Mix™ (Bio-Rad Laboratories, Hercules, CA, USA), and 6.3 µL DEPC-treated water. Validated RT-PCR oligonucleotide primer sequences of forward and reverse primers specific for target genes were as follows: *β-casein* Forward, 5'-AAATCTGCACCTTCCTCTGC-3'; *β-casein* Reverse, 5'-GAACAGGCAGGACTTTGGAC-3'; *β-actin* Forward, 5'-GCATGGAATCCTGCGGC-3'; *β-actin* Reverse, 5'-GTAGAGGTCCTTGCGGATGT-3'. RT-PCR reactions were performed by initial incubation at 95 ℃ for 3 min followed by 50 cycles of denaturation at 95 ℃ for 10 s, annealing at specific temperature for 15 s (bovine *β-casein* at 55 ℃), and extension at 72 ℃ for 30 s. RT-PCR analysis was computed using the threshold cycle (2-ΔΔCT method, Livak and Schmittgen, 2001) as a convenient way to analyze relative gene expression changes from real-time quantitative PCR experiments. Relative quantification of expression levels of target genes in a treatment group was compared with the control group (Livak and Schmittgen, 2001).

Protein Extraction and Quantification

After incubation in the treatment medium for 72 hours, the culture medium was separated from adherent cells for protein quantity quantification. Then the cells were washed twice with ice cold 1x PBS and then 200 µL cell lysis buffer containing 10 mM Tris/HCl, pH 8.3, 8 M urea, 5 mM EDTA, 4% CHAPS, and 1x protease inhibitors cocktail (GE Healthcare, Piscataway, NJ, USA) was added. The cell lysates were incubated at room temperature for 30 min and centrifuged at 14,000 rpm for 30 min at 20 ℃. Also, the culture medium was centrifuged at 300g for 5min at 4 ℃. The cell and medium supernatant was transferred to a new tube for protein quantification using Pierce BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instruction.

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Proteome Analysis

Upon completion of cell culture, cellular proteins were extracted by using cell lysis buffer containing 20mM Tris, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM EDTA, 0.1% SDS and protease inhibitor (Complete EDTA-free, Roche) after washing twice with ice cold 1x PBS. The cell lysates were incubated at 4 ℃ for 30 min and centrifuged at 13,000 g for 10 min at 4 ℃.

In proteome analysis, 100 μg of cell lysate proteins were resuspended in 0.1% SDS in 50 mM triethyl ammonium bicarbonate (TEABC, pH 8.0). The proteins were chemically denatured with 10 mM TCEP at 60 ℃ for 30 min and alkylated with 50 mM MMTS at room temperature for 30 min in the dark. Proteolytic digestion was done with trypsin (protein:trypsin = 50:1, g/g) overnight at 37 ℃. Digested peptides were desalted and concentrated before subjecting to LC-MS/MS analysis. Total peptides were analyzed by nano UPLC-mass spectrometry/electrospray ionization quadrupole time-of-flight (nano UPLC-MS/ESI-Q-TOF, Waters, Manchester, UK). LC separations of peptides were performed using nano acquity system equipped with Symmetry C18 5 μm, 5 mm × 300 μm pre-column and CSH C18 1.7 μm, 25 cm × 75 μm analytical column (Waters). The samples were separated with 3–40% gradient mobile phase B (0.1% formic acid in ACN) at 300 nL/min flow rate followed by 20 min rinse with 90% of mobile phase B. Data-dependent analysis (DDA) was performed for obtaining 2 analytical replicates for 3 biological sets. The method can read full MS scan ranging from m/z 400–1600 every 0.5 s and MS/MS scans ranging from m/z 100–1990 every 0.5 s per scan on three most intense ions in the full-scan mass spectrum. Protein identifications were assigned against IPI_bovine_database (v3.73; 30,403 entries) using MASCOT search engine v2.4 (Matrix science, UK) assuming trypsin as the digestion enzyme

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with parent ion tolerance of 0.2 Da and fragment ion mass tolerance of 0.1 Da. Two missed cleavages were allowed during trypsin digestion. Oxidation (Met) and Methylthio (Cys) were specified as variable modification. Identified proteins with probability > 95% were filtered out. For evaluation of protein identification false discovery rate, data were searched against a combined database with normal and a decoy created by MASCOT. In this study, false discovery rates for each experiment ranged at < 1%. For protein quantification, emPAI score of each protein was applied to calculate relative ratio (Ishihama et al., 2005).

Metabolome Analysis

After finishing the cell culture, the cells were collected for metabolite quantity determination as described by Park et al. (2012). First, the cells were washed twice with 5% mannitol. Then 600 uL of methanol, 200 uL of Milli-Q water and 400 uL of chloroform were added and the cell lysates were centrifuged at 10,000 g for 3min at 4 ℃. The aqueous layer was filtered to remove proteins by centrifugation using 3-kDa cut-off filter at 13,000 g/ for 120 min at 4 ℃. The filtrate was lyophilized and dissolved in 50 μL Milli-Q water.

In metabolome analysis, the metabolites were analyzed via ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS). Liquid chromatography was performed on Waters Acquity UPLC™ system (Waters, Manchester, UK) by using an Acquity HSS T3 column (1.8 μm, 2.1 × 150 mm; Waters, Manchester, UK). Column oven temperature was maintained at 35 ℃ and the auto-sampler temperature was maintained at 4 ℃. The mobile phases were (A) 5 mM tributyl ammonium acetate in water and (B) methanol. The linear gradient program began with 98% (A) for 3 min and then proceeded to 80% (B) for over 27 min and then returned to initial conditions of 98% (A) maintained for 5 min. The total cycle time was 35 min with flow rate of 300 μL/min and an

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injection volume of 5 µL using full-loop mode. Mass spectrometry was performed using a Waters Xevo™ Q-TOF/MS (Waters Corp., Manchester, UK). Ionization was performed in negative electrospray (ESI) mode. Mass range was set at m/z 50–1,000 Da with 0.3-s scan time. Conditions used for the ESI source were as follows: capillary voltage, 3.0 kV; sampling cone, 30 V; extraction cone, 3.0 V; source temperature, 100 ℃; and desolution temperature, 350 ℃. Nitrogen was used as desolvation agent in addition to cone gas with a flow rate of 500 L/h. The collision energy was set at 3 eV. LockSpray™ interface was used to ensure mass accuracy. For this, leucine-enkephalin (m/z 554.2615 in negative ionization mode) was infused at 200 pg/µL concentration at 20 µL/min flow rate.

Statistical Analysis

Statistical analysis was tested for significance using SPSS statistic software (SPSS Inc., Chicago, IL, USA). Data were analyzed using one-way analysis of variance (one-way ANOVA) and differences among treatments were determined using Duncan Multiple Range Test (DMRT). Statistical differences between two treatment groups in each experiment were determined by t-test. Mean difference was considered statistically significant at $P < 0.05$.

Up-regulated and down-regulated proteins were detected significantly using semi-quantification relative ratio (the ratio was ≥ 2 or ≤ 0.5 respectively) and detected proteins were analyzed by using web site program (http://www.pantherdb.org) for the pathway analysis (*Bos taurus*). Also, differentially expressed metabolites were detected statistically and $P < 0.05$ is considered significantly different using student t-test in comparison to control. Detected metabolites were analyzed by using MetaboAnalyst 3.0 program for the pathway analysis (*Bos taurus*).

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RESULTS AND DISCUSSION

Efficacies of different methionine types for protein synthesis were compared. How the expression levels of *β-casein* mRNA, proteins, and metabolites were affected by different forms of supplemented methionine was also elucidated.

Amino Acid Dosage and Time Sampling

L-Met dosage test showed that 0.6 mM level was the most effective concentration in increasing *β-casein* mRNA expression. The expression level of *β-casein* mRNA was continuously elevated when L-Met level was increased which peaked at 0.6 mM level (Figure 1). No further increase was observed when L-Met level was further increased to 0.9 mM. This result indicates that 0.6 mM would be the optimal level in increasing *β-casein* mRNA expression. This result agrees with the result reported by Limin et al. (2012), showing that 0.6 mM L-Met is the best concentration to increase *β-casein* protein expression.

With control at 0 mM level, the best incubation time ($P<0.05$) for *β-casein* mRNA expression level was determined to be at 72 h (Figure 2). After 72 h, no significant difference in *β-casein* mRNA expression was observed (Figure 2). In addition, the best time where protein synthesis had the highest level coincided at the point where *β-casein* mRNA expression was also at the highest level (Figure 3). This indicates that 72 h was the best incubation time for MAC-T cells to secrete β-casein protein. This outcome suggests that 72 h should be selected to test different Met isomers because of its efficacy in increasing *β-casein* mRNA expression and protein synthesis in MAC-T cells.

β-Casein Expression as well as Protein Content is Increased upon Addition of Different Isomers of Met

Upon addition of different isomers of Met (D, L and HMBi) using the obtained optimal dosage and time in the preliminary experiment, HMBi showed the highest *β-casein* relative

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expression followed by D and L-Met (Figure 4). This shows that HMBi is the most efficient in stimulating *β-casein* gene expression. On the other hand, in case of cell protein content upon addition of different isomers of Met in MAC-T cells, D-Met has the highest protein content but not significantly different with L-Met and the control ($P < 0.05$), but L-Met and the control is also not significantly different with HMBi (Figure 5).

Protein synthesis in culture medium treated with HMBi showed the highest protein synthesis but it is not significantly different with L-Met ($P < 0.05$). On the other hand, L-Met is not significantly different with D-Met group ($P < 0.05$). But D-Met group is not significantly different ($P < 0.05$) with the control (Figure 6). Lastly, the total protein synthesis in cell and medium showed that HMBi, L-Met and D-Met treatments have higher protein synthesis quantity ($P < 0.05$) than the control (Figure 7). A study in Pekin ducks reported that Met analogue has lower toxicity compared with D- and L-Met (Xie et al., 2007). The toxicity of Met and its analogues is due to transamination process leading to the production of intermediate products such as 3-methylthiopropionate that will be eventually converted to volatile sulphur compounds methanethiol and/or hydrogen sulphide (Steel and Benevenga 1978, 1979; Steel et al., 1979; Finkelstein and Benevenga, 1984, 1986). The key role of the transamination pathway in methionine toxicity has recently been confirmed (Dever and Elfarra, 2008). The methionine toxicity effect of transamination pathway has already been elaborated (Dever and Elfarra, 2008).

In general, *beta-casein* relative gene expression is increased by the addition of different isomers of Met. The result reported in the study of Limin et al. (2012) by adding 0.6 mM L-Met also increased *β-casein* contents in culture medium determined by RP-HPLC. The availability of amino acids for mammary epithelial cells is not only important for the regulation of translation, but also important for the transport rate of amino acids as one of the major limiting factors for protein synthesis (Reynolds et al., 1994; Baumrucker, 1985;

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Shennan and Peaker, 2000).

A study in the plasma kinetics of HMBi in rumen-cannulated cows by Graulet et al. (2005) by administering spot dose of 50g Met equivalent to the liquid phase of the rumen content shows that analysis of plasma concentrations of HMBi and its metabolites, HMB analogue, Met, isopropyl alcohol and acetone based on the calibration curve established by modeling the area under the curve on the response to increasing doses serving as the main end point shown that HMBi was quickly absorbed and hydrolyzed to HMB analogue and isopropyl alcohol, and then readily converted into Met and acetone.

In another study by Rulquin et al. (2006) comparing the plasma response of dairy cows to HMBi and HMB analogue supplementation showed that Met plasma concentration collected 30 min before and 2 and 6 hours after morning feeding was higher in HMBi group compared with the non-supplemented control group. But feeding the dairy cows with HMB analogue resulted in no change in plasma Met implying that HMBi is more readily absorbed and converted to Met in blood plasma.

Comprehensive Proteomics Analysis for Pathways related to Milk Protein Synthesis

D-Met, L-Met, HMBi vs Control. A total of 39 upregulated and 77 downregulated proteins, 46 upregulated and 68 downregulated proteins, and 40 upregulated and 78 downregulated proteins were observed in L-Met, D-Met and HMBi treated groups, respectively (Table 1). In addition, several proteins involved in protein and energy metabolism were also change in their expression due to the effect of adding different Met isoforms. Eukaryotic Translation Initiation Factor 3 Subunit A (EIF3A) was upregulated while Ribosomal Protein S12 (RPS12) and Ribosomal Protein S21 (RPS21) were downregulated by HMBi addition. EIF3A protein is important for stimulating protein synthesis because these genes are important transcription factors for initiation of protein translation while the protein RPS12 and RPS21 are ribosomal proteins that are components of 40S subunit. This trend, the increase in EIF3A and the

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decrease in RPS12 and RPS21 ribosomal protein components is an interesting result because previous studies reported the relationship of ribosomal proteins controlling the expression of casein genes (Bionaz et al., 2012). Previous study observed a decrease or unchanged ribosomal component expression despite the increase in milk protein synthesis especially during the induction of translation during lactation (Bionaz et. al, 2012). This may imply that the decrease or maintaining the number of ribosomal components but the increase in casein expression is a mechanism by which the mammary gland is prioritizing mRNA translation coding for proteins which are related to milk synthesis over non-milk-specific proteins like ribosomes which means the cells are now functioning to synthesize milk protein in its full capacity (Bionaz et al., 2012).

This finding was also observed on previous studies in mice and goats wherein the expression of sheep beta-globulin in mice resulted in the decrease in the expression of other milk proteins being accounted to transcriptional competition (McClenaghan et al., 1995). But interestingly, there was no observable changed in the level of total milk protein which means that the protein synthesis machinery is already working at full capacity in the lactating mammary tissues (McClenaghan et al., 1995). The protein synthesis machinery in the mammary gland is adaptably decreased during lactation to increase competitive advantage for casein synthesis. This coincides with the big increase in gene expression of genes specific for milk protein synthesis in the mammary gland during lactation mainly due to the increase transcription and longer half-life because of elongated poly-A tail (Bionaz et. al, 2012). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) which is involve in energy metabolism was upregulated by supplementing both L-Met and D-Met. On the other hand, Eukaryotic Translation Initiation Factor 4A1 (EIF4A1) was downregulated but Eukaryotic Translation Initiation Factor 4A2 (EIF4A2) was upregulated by supplementing D-Met. These two proteins are important for the initiation of protein translation.

Interestingly, GADPH enzyme translation was stimulated upon addition of L-Met and D-Met, but not by HMBi. This enzyme stimulates the important energy-yielding process of

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carbohydrate metabolism during glycolysis and also the reversible oxidative phosphorylation of the glyceraldehyde-3-phosphate in the availability of nicotinamide adenine dinucleotide and inorganic phosphate (NAD) (GAPDH, EC 1.2.1.12). (Nicholls et al., 2012).

Protein pathway analysis revealed that L-Met stimulated ATP synthesis, PI3 kinase and pyruvate metabolism while D-Met stimulated fructose-galactose metabolism, glycolysis, PI3 kinase and pyruvate metabolism. On the other hand, HMBi treatment stimulated glycolysis and pentose phosphate pathway (Table 2). This protein pathway analysis result shows that different forms of Met stimulate different pathways involve in protein and energy metabolism. Table 3 also shows the list of all protein pathways affected by treatment supplementation in comparison to control.

D-Met, L-Met vs HMBi. Using HMBi as the basis for comparison, there were 53 and 57 increased proteins for L-Met and D-Met, respectively. For decreased proteins, there were 48 and 36 in L-Met and D-Met for a total of 101 and 93 increased/decreased proteins for L-Met and D-Met groups (Table 4). In case of L-Met supplementation, there was increased in the protein related to energy synthesis such as ATP synthase subunit β, mitochondrial (ATP5B). Also there was an increased in protein expression in protein synthesis-related machinery such as Ribosomal Protein S21 (RPS21) in L-Met and the ribosomal Protein S12 (RPS12) in L-Met and D-Met treatments which are components of ribosomes. Previous studies already reported that decrease in ribosomal components but increase in casein expression is the mechanism by which the mammary epithelial cells are prioritizing mRNA translation coding for proteins related to milk synthesis over non-milk-specific proteins like ribosomes (Bionaz et al., 2012). This is reflected in the current result wherein HMBi addition has lower ribosomal components expression but higher mRNA casein expression in comparison to D- and L-Met addition. In terms of protein pathways affected by treatment supplementation, addition of L-Met caused the stimulation of ATP synthesis (Table 5).

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D-Met vs. L-Met. Conducting a t-test statistical comparison between D-Met and L-Met groups, there were 58 increased and 46 decreased proteins for a total of 104 proteins (Table 6). For D-Met group, there was decreased in proteins related to energy synthesis such as Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and ATP synthase subunit β, mitochondrial (ATP5B). In terms of protein pathways affected by D-Met supplementation, ATP synthesis was also affected (Table 7).

Comprehensive Metabolomic Analysis for Pathways Related to Milk Protein Synthesis

Intracellular Amino Acid Levels. Intracellular amino acid levels in MAC-T cells were also analyzed (Figure 8). One very distinct observation is that upon addition of different isoforms of Met, supplementation of D- and L-Met increased the concentration of Met but not in HMBi group ($P < 0.05$). This trend is also seen in case of valine, isouleucine, tyrosine and aspartatic acid. In terms of other amino acids, there was also higher concentration of leucine found inside the cells in L- and D-Met groups but D-Met group is not significantly different with HMBi group ($P < 0.05$). This was the opposite of what we expected because previous studies showed that Met and Leu shared the same transport system in the mammary gland that will entail absorption competition. Previous study identified that LAT1 (L-type AA transporter) is the primary transporter of L-Met and LAT2 (D-type AA transporter) is the primary transporter of D-Met into the mammary gland (Baumrucker, 1985). L-Met and D-Met can be also absorbed via ASCT (sodium-independent neutral and basic AA transporter system) in the mammary gland (Baumrucker, 1985). Same as D-Met and L-Met, Leu also is being absorbed in LAT1 (Kanai et al., 1998; Prasad et al., 1999), LAT2 (Rossier et al., 1999), ASCT1 (Arriza et al., 1993; Zerangue and Kavanaugh, 1996) as well as ASCT2 transport system (Utsunomiya-Tate et al., 1996; Kekuda et al., 1996). Although Met and Leu usually competes for the same amino acid transporter, Leu has also its own alternative transporter specifically y+LAT1 AmAT-L-lc which is a neutral amino acid transporter that transports Leu

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but not Met (Torrents et al., 1998; Pfeiffer et al., 1999a; Kanai et al., 2000) So more investigations should be done to gather data on several AA transporters with regards to their affinity and mechanism of transport (Shennan and Boyd, 2014).

D-Met, L-Met, HMBi vs Control. Table 8 displays the list of pathway detection metabolites. Most of pathways related to detected metabolite were involved in energy and amino acid metabolism. Based on metabolite pathway analysis, both D- and L-Met supplementation stimulated amino acid metabolism pathways such as alanine, aspartate and glutamate metabolism, aminoacyl-tRNA biosynthesis, arginine and proline metabolism, beta-alanine metabolism, cysteine and methionine metabolism, D-glutamine and D-glutamate metabolism, glycine, serine and threonine metabolism, phenylalanine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis and valine, leucine and isoleucine biosynthesis pathway as well as energy metabolism pathways such as glycolysis or gluconeogenesis pathway and pyruvate metabolism. On the other hand, supplementation of L-Met alone influenced amino acid metabolism pathways such as histidine metabolism and valine, leucine and isoleucine degradation pathway as well as energy metabolism related-pathways such as butanoate metabolism, citrate cycle (TCA cycle) and glyoxylate and dicarboxylate metabolism pathways. In case of supplementing D-Met, it influenced pentose phosphate pathway which is related to energy metabolism.

Results of metabolite analysis showed that L-Met supplementation caused increases of 11 metabolites, D-Met supplementation caused increases of 7 metabolites and decreased of UMP, and HMBi supplementation caused increases of 3 metabolites and decreased of UMP and N-acetyl-L-glutamate ($P < 0.05$) (Table 9).

Addition of different isoforms of Met stimulated the production of intermediate metabolites for energy production (Table 9). Supplementation of L-Met stimulated the production of energy metabolites such as pyruvate, malate, and fumarate, well-known as

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intermediates of Krebs cycle. On the other hand, HMBi supplementation caused increases of energy metabolite glucose 1-phosphate. Previous researches have shown that energy intermediates could stimulate protein synthesis indirectly by inhibiting AMPK signal (Sarbassov et al., 2005), a known direct inhibitor of mTOR pathway. The adenosine monophosphate kinase (AMPK) complex is a phylogenetically conserved fuel-sensing enzyme which is present in all mammalian cells (Richter and Ruderman, 2009). AMPK may modulate if ever there was a decreased in energy status, consequently inhibiting very high energy consuming protein synthetic process (Proud et al., 2007).

D-Met, L-Met vs HMBi. Considering the detected metabolite, there was an increased in Met concentration level inside the cell when D- and L-Met isoforms were supplemented in comparison to HMBi supplementation (Table 10). There was also increased in energy-related metabolites such as glycerol-3-phosphate, pyruvate, fumarate, citrate, phosphoenol pyruvate, NADPH and FAD in L-Met supplemented group. In case of D-Met supplemented group, there was increased in energy-related metabolites such as lactate, NAD+, citrate and phosphoenol pyruvate but a decreased in fructose-6-phosphate.

D-Met vs. L-Met. Again, looking at the detected metabolite data, there was increased in amino acids serine, alanine, glutamine, isoleucine and aspartate but the decreased of energy metabolites such as fructose-6-phosphate, glycerol-3-phosphate, UMP, guanosine monophosphate (GMP) and adenosine monophosphate (AMP) in D-Met supplemented group compared to L-Met supplemented group (Table 11).

To summarize interactions of the addition of different isoforms of Met in MAC-T cells, diagrams were created to demonstrate different relationships among various components (Figures 9, 10, and 11). L-Met supplementation stimulated PI3 kinase and GAPDH as well as pyruvate metabolism and ATP synthesis pathways. Metabolites such as pyruvate, malate, and fumarate were also stimulated. These metabolites are involved in energy production pathway

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(Figure 9). These pathway and metabolite stimulations may promote the stimulation of mTOR activity that elevated *β-casein* expression that will eventually increase protein synthesis.

On the other hand, the addition of D-Met stimulated PI3 kinase and GAPDH enzymes, pyruvate metabolism, glycolysis and galactose metabolism (Figure 10), all of which are energy production pathways that may inhibit the AMPK which is a known inhibitor of mTOR, thus, promoting *β-casein* gene expression that could increase protein synthesis.

Lastly, HMBi addition stimulated EIF3A protein important for protein translation initiation. HMBi also stimulated glycolysis and pentose phosphate pathways which are energy production metabolic pathways and the increased of glucose 1-phosphate, an energy metabolic intermediate product (Figure 11), all of which could influenced mTOR stimulation thus the increased in *β-casein* gene expression resulted to increased protein synthesis.

CONCLUSION

Results of this study showed that HMBi-treated group exhibited much better performance in stimulating the expression of *β-casein* mRNA. HMBi supplementation stimulated proteins and metabolites involved in energy and protein synthesis. In addition, protein and metabolic pathways involved in protein and energy synthesis were upregulated. As a result, HMBi-treated group resulted in higher protein concentration in MAC-T cell medium although not different with L-Met treatment medium protein ($P < 0.05$). But it is also important to note that there was no difference also in terms of protein synthesis efficiency using the D- and L-Met isoforms. Both the D- and L-isoforms has the same medium protein concentration and *β-casein* mRNA expression higher than the control ($P < 0.05$). In conclusion, HMBi is the most effective isoform to be utilized to increase protein synthesis in bovine mammary epithelial cells in vitro. Also the D- and L-Met isoforms can be used alternatively without any significant change in protein synthesis efficiency.

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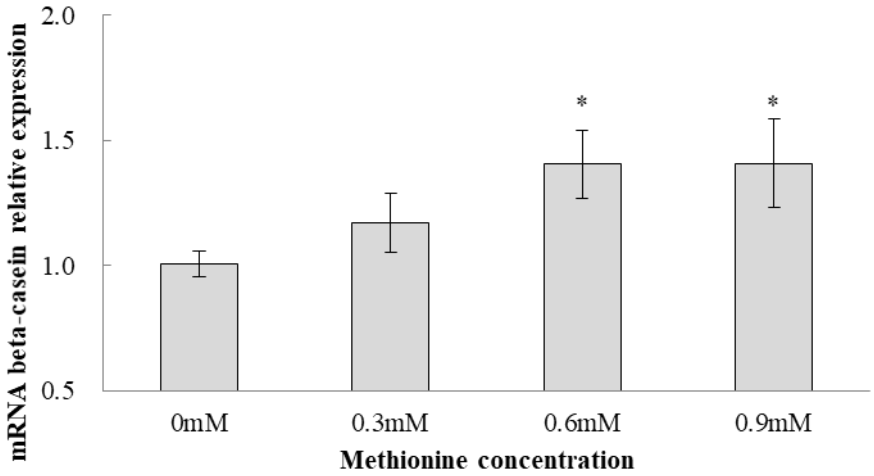

Jeon. Figure 1. *β-casein* mRNA expression level in MAC-T cells incubated with different levels of L-methionine (0, 0.3, 0.6, 0.9 mM) for 24 h. Values are expressed as means ± SE (n = 6 per group). * Indicates significant difference in comparison to control (0 mM/L) of L-methionine ($P < 0.05$, student's t-test)

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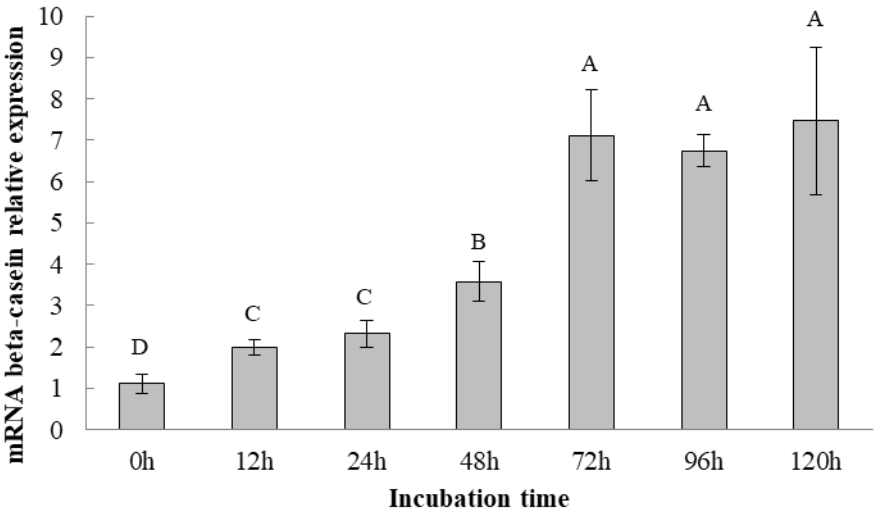

Jeon. Figure 2. *β-casein* mRNA expression level in MAC-T cells incubated with 0 mM L-methionine at different time points (0, 24, 48, 72, 96, 120 h). Values are expressed as means ± SE (n = 6 per group) and A, B, C, D indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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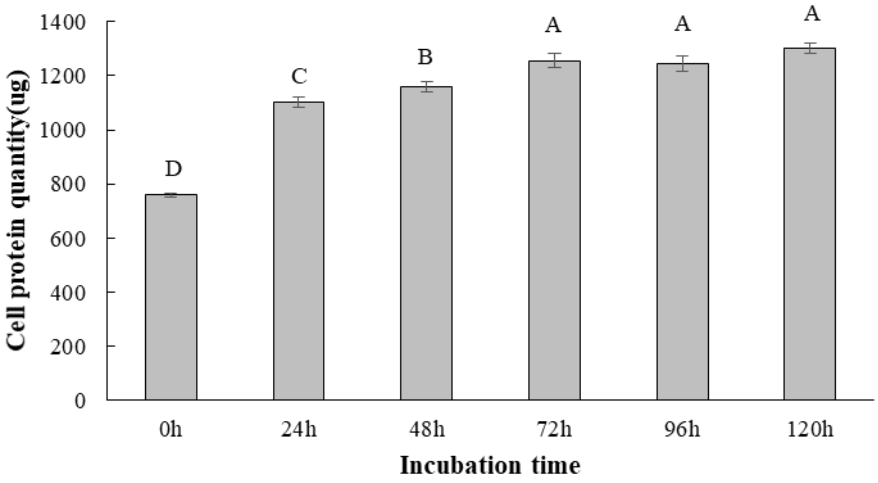

Jeon. Figure 3. Cell protein quantity in MAC-T cells incubated with 0 mM L-methionine at different time points (0, 24, 48, 72, 96, 120 h). Values are expressed as means ± SE (n = 6 per group) and A, B, C, D indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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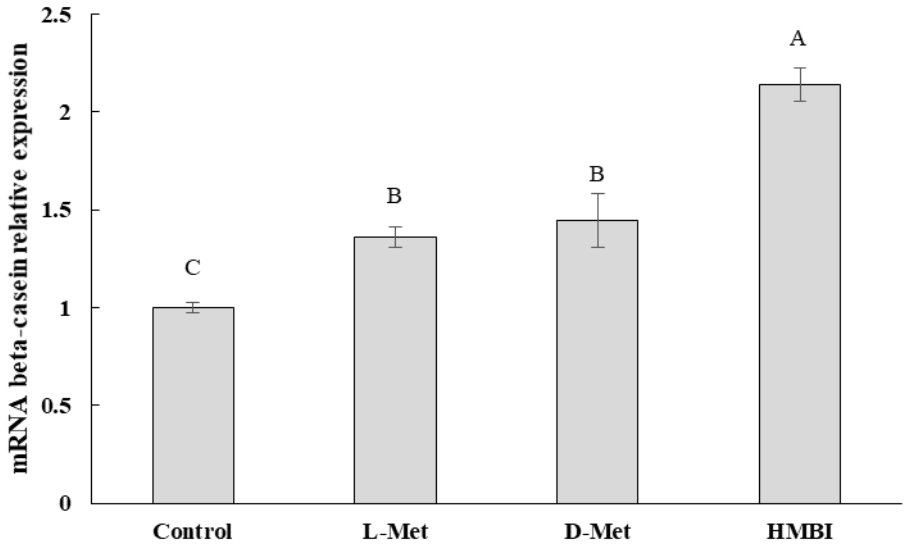

Jeon. Figure 4. . *β-casein* mRNA expression level in MAC-T cells incubated with control (0 mM), L-methionine (0.6 mM), D-methioinine (0.6 mM) and 2-hydroxy-4-methylthiobutanoic acid I (HMBi) (0.6 mM). Values are expressed as means ± SE (n = 6 per group) and A, B, C indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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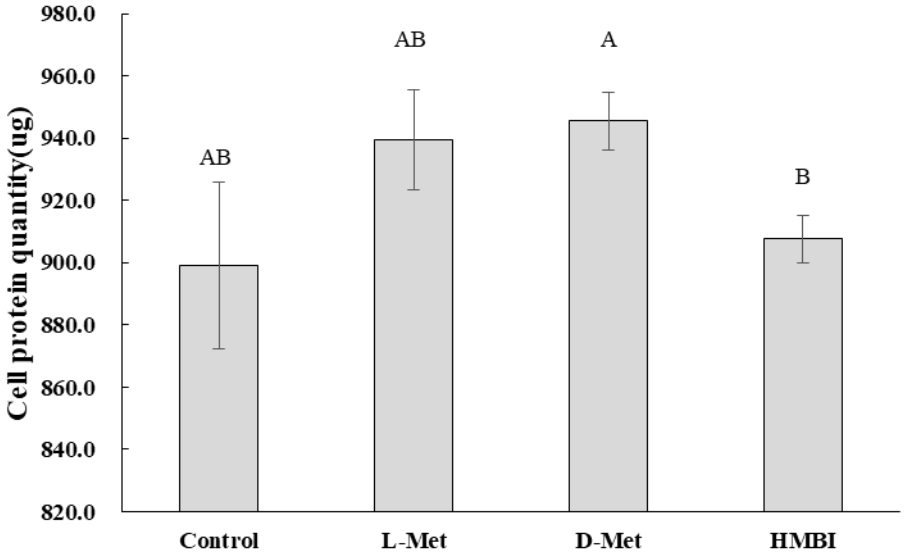

Jeon. Figure 5. Cell protein quantity in MAC-T cells incubated with control (0 mM), L-methionine (0.6 mM), D-methionine (0.6 mM) and 2-hydroxy-4-methylthiobutanoic acid I (HMBi) (0.6 mM). Values are expressed as means ± SE (n = 6 per group) and A, B indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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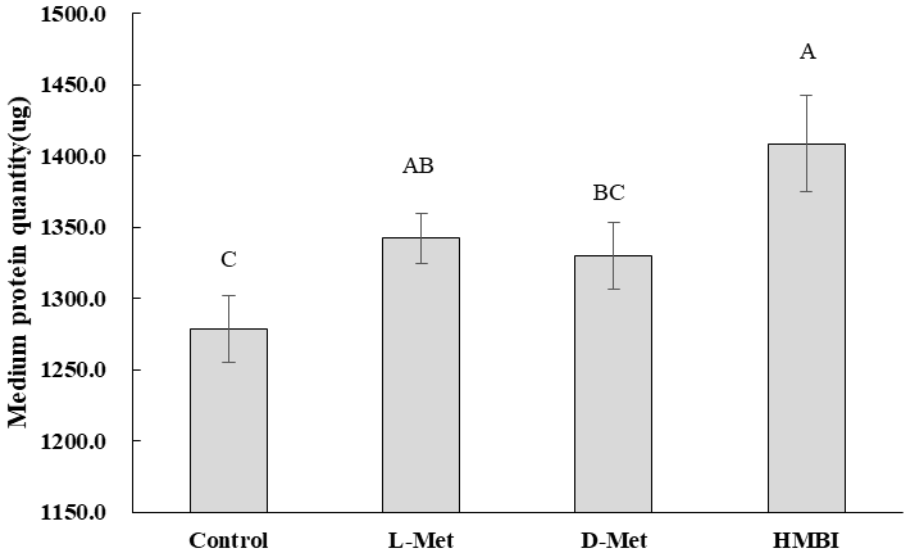

Jeon. Figure 6. Medium protein quantity in MAC-T cells incubated with control (0 mM), L-methionine (0.6 mM), D-methionine (0.6 mM) and 2-hydroxy-4-methylthiobutanoic acid I (HMBi) (0.6 mM). Values are expressed as means ± SE (n = 6 per group) and A, B, C indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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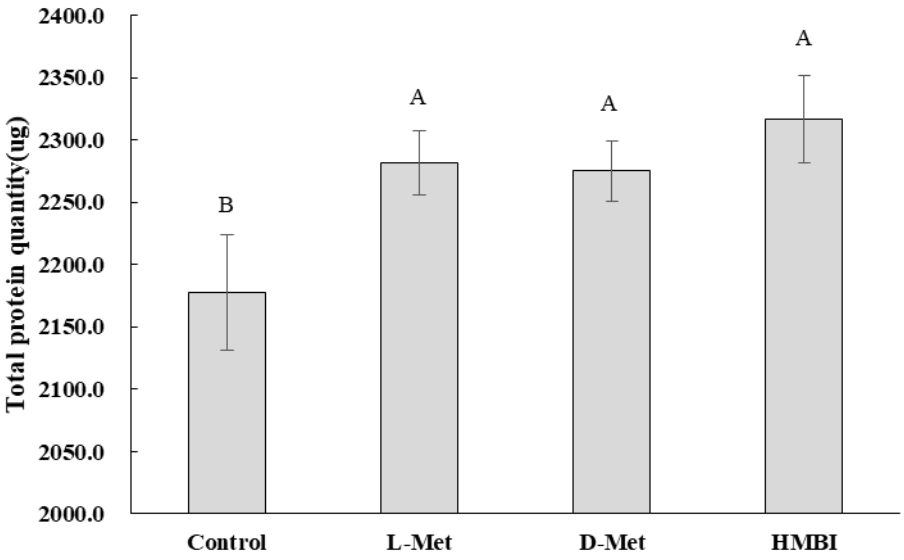

Jeon. Figure 7. Total protein quantity (cell and medium) in MAC-T cells incubated with control (0 mM), L-methionine (0.6 mM), D-methionine (0.6 mM) and 2-hydroxy-4-methylthiobutanoic acid I (HMBi) (0.6 mM). Values are expressed as means ± SE (n = 6 per group) and A, B indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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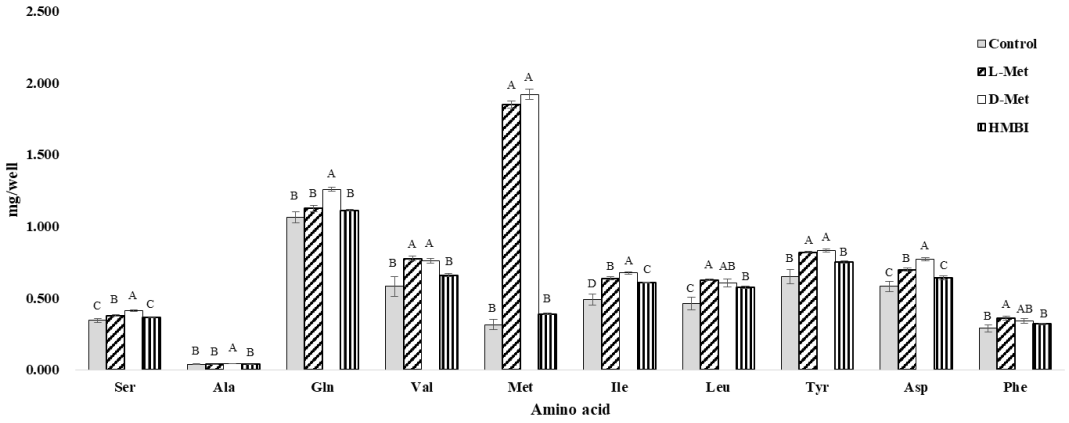

Jeon. Figure 8. Amino acids in MAC-T cells incubated with control (0 mM), L-methionine (0.6 mM), D-methionine (0.6 mM) and 2-hydroxy-4-methylthiobutanoic acid I (HMBi) (0.6 mM). Values are expressed as means ± SE (n = 3 per group) and A, B, C, D indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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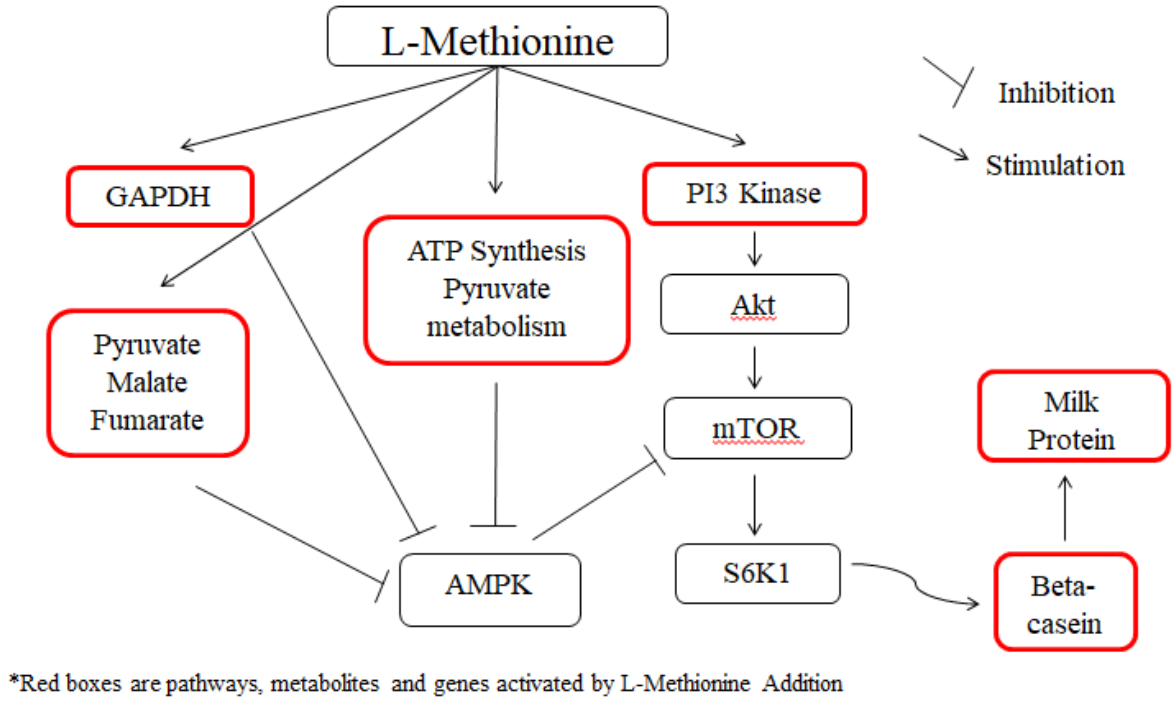

Jeon. Figure 9. Diagram showing the effect of L-methionine on protein synthesis pathway.

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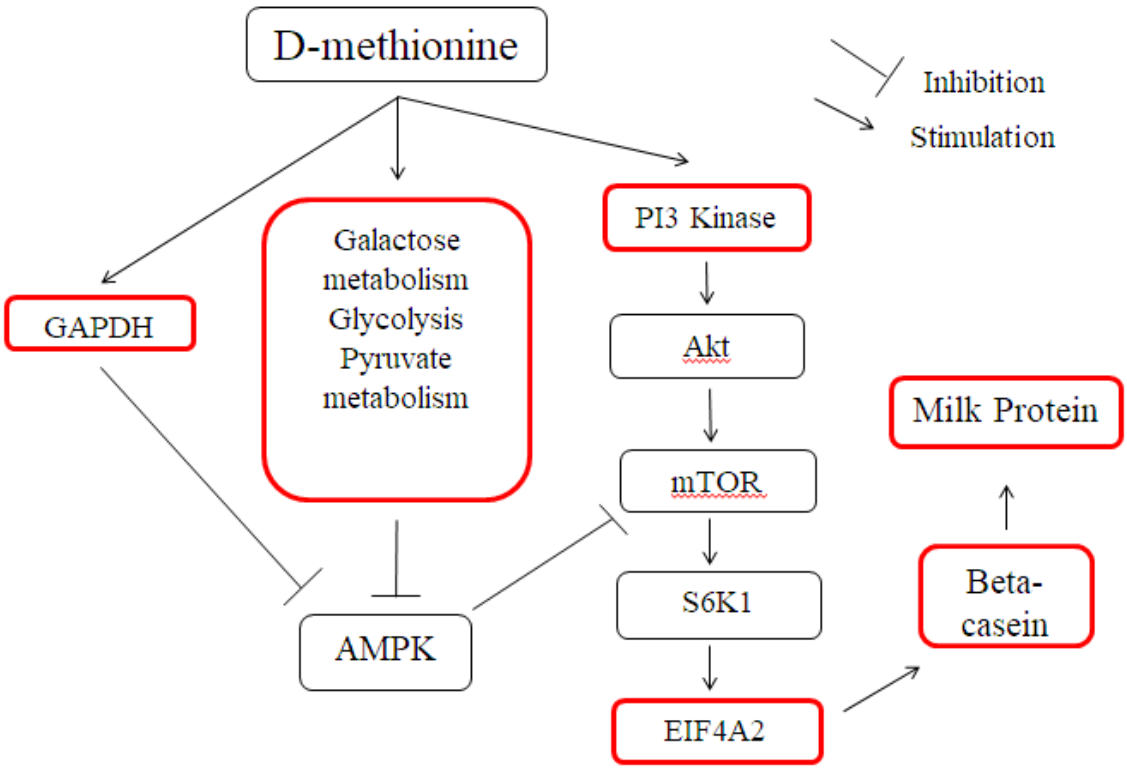

Jeon. Figure 10. Diagram showing the effect of D-methionine on protein synthesis pathway.

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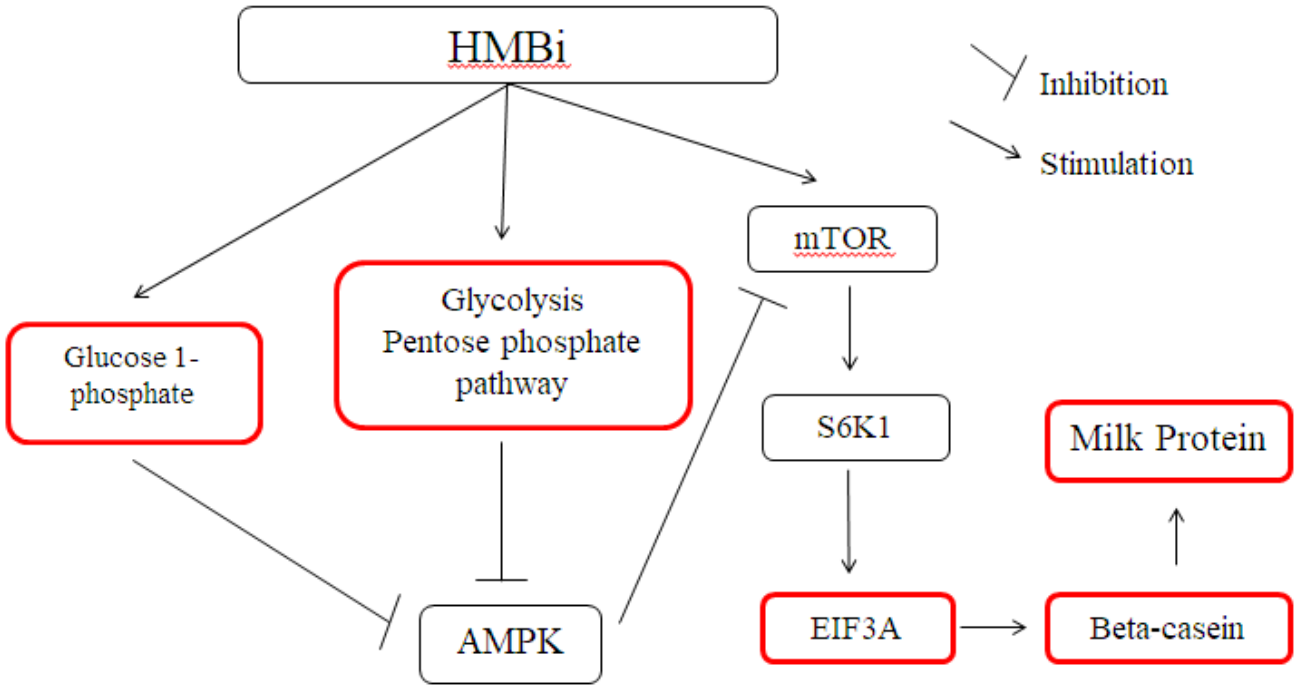

*Red boxes are pathways, metabolites, proteins and genes activated by HMBi Addition

Jeon. Figure 11. Diagram showing the effect of 2-hydroxy-4-methylthiobutanoic acid I (HMBi) on protein synthesis pathway.

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Table 1. List of differently expressed proteins in MAC-T Cells (in comparison to control)

Detection Protein	**L-Met[1]**	**D-Met[2]**	**HMBi[3]**
Increasing Number	39	46	40
Decreasing Number	77	68	78
Total Protein Number	116	114	118
List of Selected Downregulated and Upregulated Proteins			
Eukaryotic Translation Initiation Factor 3 Subunit A (EIF3A)			▲
Ribosomal Protein S21(RPS21)			▼
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	▲	▲	
EIF4A1 (Eukaryotic Translation Initiation Factor 4A1)		▼	
EIF4A2 (Eukaryotic Translation Initiation Factor 4A2)		▲	
ATP5A1 (ATP Synthase, H+ Transporting, Mitochondrial F1 Complex, Alpha Subunit 1)	▼		
RPS12 (Ribosomal Protein S12)	▼		▼

Selection Criteria: Upregulated, > 2-fold in protein expression vs. Control, Downregulated: < 0.5-fold in protein expression vs. Control.

[1] L-Met = L-methionine

[2] D-Met = D-methionine

[3] HMBi = 2-hydroxy-4-methylthiobutanoic acid I

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Table 2. List of protein pathways related to protein and energy metabolism affected by supplementation (in comparison to control)

Detected Pathway	L-Met[1]	D-Met[2]	HMBi[3]
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 kinase/MAP kinase cascade		●	
Insulin/IGF pathway -protein kinase B signaling cascade		●	

Pathway analysis (*Bos taurus*). The analysis used increasing detected pathway at $P < 0.05$.

[1] L-Met = L-methionine

[2] D-Met = D-methionine

[3] HMBi = 2-hydroxy-4-methylthiobutanoic acid I

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Table 3. List of all protein pathways affected by supplementation (in comparison to control)

Detected Pathway	L-Met[1]	D-Met[2]	HMBi[3]
5HT2 type receptor mediated signaling	●	●	●
Alzheimer disease-presenilin	●	●	●
Apoptosis signaling			●
ATP synthesis	●		
Cadherin signaling	●	●	●
CCKR signaling map	●	●	●
Cholesterol biosynthesis	●	●	●
Cytoskeletal regulation by Rho GTPase	●	●	●
De novo purine biosynthesis	●	●	●
De novo pyrimidine deoxyribonucleotide biosynthesis	●	●	●
De novo pyrimidine ribonucleotides biosythesis	●	●	●
EGF receptor signaling	●	●	●
FAS signaling		●	●
FGF signaling	●	●	●
Fructose galactose metabolism		●	
Glycolysis		●	●
Gonadotropin-releasing hormone receptor	●	●	●
Heterotrimeric G-protein signalling pathway -Gialphaand G s alpha mediated	●		●
HeterotrimericG-protein signalingpathway -Gq alpha and Go alpha mediated	●		●
Histamine H1 receptor mediated signaling	●	●	●
Huntington disease	●	●	●
Inflammation mediated by chemokine and cytokine signalling	●	●	●

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Integrin signalling	●	●	●
Nicotinic acetylcholine receptor signaling	●	●	●
Oxytocin receptor mediated signaling	●	●	●
p53	●	●	●
Parkinson disease	●	●	●
Pentose phosphate			●
PI3 kinase	●	●	
Pyruvate metabolism	●	●	
Salvage pyrimidine ribonucleotides	●	●	●
Thyrotropin-releasing hormone receptor signaling	●	●	●
Wnt signaling	●	●	●
Ubiquitin proteasome			●
Insulin/IGFpathway-mitogen activated protein kinase kinase/MAP kinase cascade		●	
Insulin/IGF pathway -protein kinase B signaling cascade		●	

Pathway analysis (Bos taurus). The analysis used increasing detected pathway at $P < 0.05$.

[1] L-Met = L-methionine

[2] D-Met = D-methionine

[3] HMBi = 2-hydroxy-4-methylthiobutanoic acid I

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Table 4. List of differently expressed proteins in MAC-T Cells (in comparison to HMBi[3])

Detection Protein	**L-Met[1]**	**D-Met[2]**
Increasing Number	53	57
Decreasing Number	48	36
Total Protein Number	101	93
List of Selected Downregulated and Upregulated Proteins		
Ribosomal Protein S21(RPS21)	▲	
ATP Synthase, H+ Transporting, Mitochondrial F1 Complex, Alpha Subunit 1 (ATP5A1)	▼	
Ribosomal Protein S12 (RPS12)	▲	▲
ATP synthase subunit β, mitochondrial (ATP5B)	▲	

Selection Criteria: Upregulated, > 2-fold in protein expression vs. HMBi, Downregulated: < 0.5-fold in protein expression vs. HMBi.

[1] L-Met = L-methionine

[2] D-Met = D-methionine

[3] HMBi = 2-hydroxy-4-methylthiobutanoic acid I

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Table 5. List of all protein pathways affected by treatment supplementation (in comparison to HMBi[3]).

Detected Pathway	L-Met[1]	D-Met[2]	Both
ATP synthesis	●		●
Cytoskeletal regulation by Rho GTPase	●		
Huntington disease			●
Parkinson disease	●		

Pathway analysis (*Bos taurus*). The analysis used increasing detected pathway at $P < 0.05$.

[1] D-Met = D-methionine

[2] L-Met = L-methionine

[3] HMBi = 2-hydroxy-4-methylthiobutanoic acid I

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Table 6. List of differently expressed proteins in MAC-T Cells (in comparison to L-Met[1])

Detection Protein	**D-Met[2]**
Increasing Number	58
Decreasing Number	46
Total Protein Number	104
List of Selected Downregulated and Upregulated Proteins	
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	▼
ATP synthase subunit β, mitochondrial (ATP5B)	▼

Selection Criteria: Upregulated, > 2-fold in protein expression vs. L-Met,

Downregulated: < 0.5-fold in protein expression vs. L-Met.

[1] L-Met = L-methionine

[2] D-Met = D-methionine

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Table 7. List of all protein pathways affected by treatment supplementation (in comparison to L-Met[2]).

Detected Pathway	D-Met[1]
ATP synthesis	●
Huntington disease	●
Cytoskeletal regulation by Rho GTPase	●

Pathway analysis (*Bos taurus*). The analysis used increasing detected pathway at $P < 0.05$.

[1] D-Met = D-methionine

[2] L-Met = L-methionine

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Table 8. List of pathway detection metabolites affected by application of treatment (compared to control)

Metabolite related pathway	L-Met[1]	D-Met[2]	HMBi[3]
Alanine, aspartate and glutamate metabolism	●	●	
Aminoacyl-tRNA biosynthesis	●	●	●
Arginine and proline metabolism	●	●	
Beta-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	●	●	
Phenylalanine, tyrosine and tryptophan biosynthesis	●	●	
Propanoate metabolism			
Pyruvate metabolism	●	●	
Tyrosine metabolism	●	●	
Ubiquinone and other terpenoid-quinone biosynthesis	●		
Valine, leucine and isoleucine biosynthesis	●	●	●
Valine, leucine and isoleucine degradation	●		●

Metabo Analyst 3.0 was used to perform the pathway analysis (*Bos taurus*). The analysis used increasing detection metabolite at $P < 0.05$.

[1] L-Met = L-methionine

[2] D-Met = D-methionine

[3] HMBi = 2-hydroxy-4-methylthiobutanoic acid I

Table 9. List of detected metabolite affected by application of treatment (compared to control)

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SUPPLEMENTING D-TYPE AND L-TYPE METHIONINE AND ITS PRECURSOR

Category	**L-Met[1]**	**D-Met[2]**	**HMBi[3]**
Increasing Metabolite List	Pyruvate	Serine	Glucose 1-phosphate
	2-Oxoglutarate	Glutamine	6-Phosphogluconate
	Fumarate	Met	Ile
	N-acetyl-L-glutamate	Aspartate	
	Malate	Ile	
	Aspartate	Leu	
	Met	Tyrosine	
	Leu		
	Tyrosine		
	Ile		
	Acetyl-Glutamic Acid		
Decreasing Metabolite List		UMP	UMP
			N-acetyl-L-glutamate

The Detected Metabolite was selected by Student's T-test (Control vs. Treatment, $P < 0.05$).

[1] L-Met = L-methionine

[2] D-Met = D-methionine

[3] HMBi = 2-hydroxy-4-methylthiobutanoic acid I

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Table 10. List of Detected Metabolite (HMBi[3] as control).

Category	L-Met[1]	D-Met[2]
Increasing Metabolite List	Serine Valine	Serine Alanine
	Methionine	Glutamine
	Leucine Tyrosine Aspartate	Valine Methionine Isoleucine
	Phenylalanine	Tyrosine
	Glycerol 3-Phosphate	Aspartate
	Pyruvate NAD+ UMP AMP Acetyl – Glutamic Acid 2-oxoglutaric acid	Lactate NAD+ UMP Pantothenate UDP N-acetylglucosamine Citrate
	Fumarate	Phosphoenol pyruvate
	Citrate Phosphoenol pyruvate UDP-glucuronic acid NADPH	
	FAD	
Decreasing Metabolite List		Fructose-6-Phosphate

The Detected Metabolite was selected by Student's T-test (HMBi vs. Treatments, $P < 0.05$).

[1] L-Met = L-methionine

[2] D-Met = D-methionine

[3] HMBi = 2-hydroxy-4-methylthiobutanoic acid I

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Table 11. List of Detected Metabolite (L-Met[2] as control).

Category	D-Met[1]	p-value
Increase Metabolite List	Serine	0.0060
	Alanine	0.0023
	Glutamine	0.0060
	Isoleucine	0.0470
	Aspartate	0.0084
Decrease Metabolite List	Fructose-6-Phosphate	0.0348
	Glycerol-3-Phosphate	0.0060
	UMP	0.0085
	GMP	0.0353
	AMP	0.0143
	Acetyl-glutamic acid	0.0330

The Detected Metabolite was selected by Student's T-test (D-Met vs. L-Met, $P < 0.05$).

[1] D-Met = D-methionine

[2] L-Met = L-methionine