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Supplementing conjugated and non-conjugated L-methionine and acetate alters expression patterns of β-casein, proteins, and metabolites related to protein synthesis in bovine mammary cells

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The study was done to determine the effect of supplementing different forms of L-methionine (L-Met) and acetate on protein synthesis in immortalized bovine mammary epithelial cell line (MAC-T Cell): Control, L-Met, conjugated L-Met and acetate (CMA), and non-conjugated L-Met and Acetate (NMA). Protein synthesis mechanism was determined by omics method. NMA group had the highest protein content in the media and β-casein mRNA expression levels ($P < 0.05$). The number of upregulated and downregulated proteins observed were 77 and 62 in CMA group and 50 and 81 in NMA group from 448 proteins, respectively ($P < 0.05$). NMA and CMA treatments stimulated pathways related to protein and energy metabolism ($P < 0.05$). Metabolomic analysis also revealed CMA and NMA treatments resulted in increases of several metabolites ($P < 0.05$). In conclusion, NMA treatment increased protein concentration and expression level of β-casein mRNA in MAC-T cells compared to CMA.

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SUPPLEMENTING CONJUGATED L-METHIONINE AND ACETATE (CMA)

INTRODUCTION

Protein synthesis requires protein synthesis machinery components and availability of amino acids as well as the supply of energy (Bionaz, Hurley, & Loor, 2012). The availability of AA is important for protein synthesis (Doepel et al., 2004; Griinari et al., 1997). AAs are anabolic factors that can induce protein gain by stimulating gene expression and mRNA translation (Lu, Li, Huang, & Gao, 2013) through mTOR pathway (Bionaz et al., 2012), which is a serine/threonine protein kinase which is a central regulator of cellular metabolism, growth and survival in response to hormones, growth factors, nutrients, energy and stress signal, that will eventually affect the expression of genes related to milk protein synthesis (Bruhat et al., 1997; Deval et al., 2009). Additionally, AAs are "building blocks" of proteins. Many past studies have shown that AA can directly regulate the expression of genes related to protein synthesis through nutrient/gene interaction (Deval et al., 2009; Jousse et al., 2004). Nan et al. (2014) have found that adding supplemental Met and Lys into bovine mammary epithelial cells using in vitro experiment design has a strong nutrigenomic effect by significantly increasing expression of genes coding for major milk protein components (i.e. casein) as well as genes related to protein translation (*ELF5*, *MTOR*) and JAK-STAT signaling (*JAK2*, *STAT5*).

In general, Met is considered as the most limiting AA for protein production in lactating dairy cows (National Research Council, 2001). Met is also an essential AA that has a major role in protein synthesis and several other biochemical and cellular processes (Metayer et al., 2008; Orellana et al., 2007).

In addition, acetate is the chief source of energy for mammary gland of ruminant animals (Sabine & Johnson, 1963). The positive effect of dietary energy on protein synthesis is partly due to the production of intracellular energy transfer molecules such as ATP, GTP,

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NADH, and NADPH in the presence of energetic precursors (Bionaz et. al, 2012; Appuhamy, Knoebel, Nayananjalie, Escobar, & Hanigan, 2012). Another important function of energy, specifically glucose, is through increased secretion of insulin due to greater dietary energy. Schmidt (1966) has shown a positive role of insulin in mammalian protein synthesis.

N-Acetyl-L-Methionine (CMA) is a rumen-protected methionine in which L-Met is conjugated with acetate. It can be separated into L-Met and acetate in small intestine or liver. It has been shown that CMA itself is more effective than L-Met alone in mammalian synthesis because CMA has higher bioavailability in ruminants after being broken down in the intestine and in the liver (Fagundes et al., 2016; Toledo, et. al. 2017). In general, rumen-protected methionine (RPM) has been developed as a product that can significantly increase protein content in milk (Rulquin & Delaby, 1997; Socha et al., 2005). In most studies, protein content is increased when RPM is supplemented (Berthiaume et al., 2006; Chilliard & Doreau, 1997; Kowalski, Pisulewski, & Gorgulu, 2003; Leonardi, Stevenson, & Armentano, 2003; Rulquin & Delaby, 1997). However, CMA and non-conjugated L-Met + Acetate as digested forms of CMA have not been yet studied for their milk protein synthesis efficiency in mammary epithelial cells and regulation of mechanism of milk protein synthesis.

Therefore, the objectives of this study were: (1) to compare the efficacy of CMA and its digested forms, L-Methionine (L-Met) and non-conjugated form (NMA; digested form of CMA), on protein synthesis using immortalized bovine mammary epithelial cell line (MAC-T Cell), and (2) to elucidate the mechanism of protein synthesis.

MATERIALS AND METHODS

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Amino Acid Dosage and Sampling Time

Immortalized mammary epithelial cell line (MAC-T) (University of Vermont, Burlington, VT, USA) were seeded into 10-cm dishes (TPP, Trasadingen, Switzerland). These cells were then seeded into BD Falcon™ 6-well multiwall plate (Franklin Lakes, NJ, USA). They were 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% CO_2 incubator (Wang et al., 2014; Wang et al., 2015).

Lactogenic DMEM/F12 differentiation medium (without FBS) was used to differentiate MAC-T cells into *β-casein* secreting cells for 72 h. 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 this 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. When MAC-T Cells reached 90% confluence, DMEM/F12 basic medium was replaced with lactogenic DMEM/F12 differentiation medium.

A preliminary study was conducted to determine effects of different concentrations of supplemental Met and culture time on *β-casein* mRNA expression. Additional L-Met (0 (as control), 0.3, 0.6, 0.9 mM) was added to lactogenic medium without FBS. Cells were harvested at 24 hours after treatment. To evaluate the effect of culture time of supplementing methionine on *β-casein* secretion of MAC-T Cells, the cells were harvested at 0, 12, 24, 36, 48, 72, 96, and

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120 h after incubated in control medium. Each treatment was performed with six replicates. MAC-T cells were collected by detaching with trypsin (Lonza, Verviers, Belgium) after washing twice with 1x PBS. Resultant data were used to determine the best concentration and culture time for supplemental Met treatments. After the preliminary study, the determined best dosage and time was used uniformly for all treatments: L-Met and acetate (Sigma-Aldrich Corp., St. Louis, MO, USA) and CMA (CJ CheilJedang Corp., Suwon, Republic of Korea). Each treatment was assigned with six replicates.

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 measured using NanoDrop 1000® Spectrophotometer with RNA-40 module (Thermo Fisher Scientific, Wilmington, DE, USA). Then cDNA was prepared using iScript cDNA synthesis kit (BioRad) according to the manufacturer's instructions. After incubating at 25 ℃ for 5 min, 42 ℃ for 30 min, and 85 ℃ for 5 min, the cDNA was quantified by using ssDNA-33 module of Thermo NanoDrop 1000® Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Real-Time PCR

Real-Time PCR (RT-PCR) analysis was performed with a total volume of 20 μL in 96-well microwell plates and a T100™ Thermal Cycler System. *β-actin* was used as a reference gene. The reaction mixture contained 50 ng cDNA, 0.6 μL forward primer, 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

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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 to the untreated group (Livak & Schmittgen, 2001).

Protein Extraction and Quantification

After incubation in the treatment medium for 72 hours, the culture medium was collected from adherent cells for protein quantity determination. 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/ 30 min at 20 ℃. Also, the culture medium centrifuged at 300g/ 5min at 4 ℃. The supernatant of cell and medium 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.

Proteomic Analysis

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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, 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/ 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 darkness. Proteolytic digestion was done with trypsin (protein:trypsin = 50:1, g/g) overnight at 37 ℃. Digested peptides were desalted and concentrated before 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 flow rate of 300 nL/min, 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 with a parent ion tolerance of 0.2 Da and a fragment ion mass tolerance of 0.1 Da. Two missed cleavages were allowed during

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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 (Ishihama, et al., 2005) of each protein was applied to calculate relative ratio.

Metabolomic Analysis

After finishing the cell culture, the culture medium was removed from the adherent cells, and the cells were used 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/ 3min at 4 ℃. The aqueous layer was filtered to remove proteins by centrifugation using 3-kDa cut-off filter at 13,000 g/ 120min 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 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 a 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 desolvation temperature, 350 ℃. Nitrogen was used as desolvation and 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 (protein quantification data, n = 6; proteomics and metabolomics data, n = 3; quantitative real-time PCR data, n = 6) were 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 or down-regulated proteins were detected significantly using semi-quantification relative ratio (the ratio was ≧2 or ≦0.5) and detected proteins were analyzed by using web site program (http://www.pantherdb.org) for the pathway analysis (*Bos taurus*). Also,

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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*).

RESULTS AND DISCUSSION

Efficacies of CMA and NMA 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. Previous researches have reported that adding encapsulated forms of L-Met into dairy diet can increase protein (Armentano & Bertics, 1993;

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Robert, Sloan, & Bourdeau, 1994; Rulquin & Delaby, 1994a, b) or increase both milk yield and protein (Illg, Sommerfeldt, & Schingoethe, 1987).

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, peaking at 0.6 mM level. No further increase was observed when L-Met level was further increased to 0.9 mM (Figure 1). This result indicates that 0.6 mM would be the optimal level. This level is in accordance 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 found to be 72 h (Figure 2). Beyond 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 outcome suggests that 72 h should be selected to test different L-Met types 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 Forms of L-Met

The following are the results obtained by the determined optimal dosage and time in MAC-T cells in the above-mentioned experiment. Upon addition of different forms of L-Met, NMA showed the highest *β-casein* mRNA relative expression followed by CMA and L-Met

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(Figure 4). This shows that NMA is the most efficient in stimulating *β-casein* gene expression.

Although there was no significant differences among treatments in cell protein content upon addition of different forms of L-Met in MAC-T cells, all the treatments increased protein synthesis numerically. However, protein synthesis tended to increase ($P < 0.10$) (Figure 5).

Protein synthesis in culture medium with NMA treatment showed the highest protein synthesis rate ($P < 0.05$). L-Met group had the second highest level of protein synthesis rate compared to the control ($P < 0.05$). But, CMA only tended to increase ($P < 0.10$) (Figure 6). On the other hand, total protein synthesis in cell and medium with NMA treatment showed the highest protein synthesis quantity ($P < 0.05$). L-Met and CMA had the second highest levels of protein synthesis quantity compared to the control ($P < 0.05$) (Figure 7). NMA had the highest protein synthesis quantity in overall total protein synthesis in MAC-T cells and culture medium than that of the control.

In general, protein synthesis is increased by the addition of different forms of L-Met especially in the culture medium where casein protein was secreted outside the cell. 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 (Baumrucker, 1985; Reynolds, Harmon, & Cecava, 1994; Shennan & Peaker, 2000).

It is known that energy availability is also a crucial factor for the synthesis of other milk components, especially milk proteins (Reynolds et al., 1994). Thus, acetate was also added as experimental treatment. Interestingly, NMA group showed higher protein synthesis than CMA conjugated group (Fig. 6). Both treatments contained the same components. The only difference was their conjugation form. This was validated by the analysis of metabolic pathway.

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Based on this analysis, CMA treatment stimulated pyruvate metabolism pathway, indicating that acetate group conjugated in CMA could not be utilized as an energy source in MAC-T cells. Consequently, cells have to produce energy via the pyruvate pathway (Pietrocola, Galluzzi, Bravo-San Pedro, Madeo, & Kroemer, 2015). Not cleaving L-Met and acetate into separate groups could cause some problem in the incorporation of L-Met into elongating peptides because ribosomes can discriminate amino acid structure within its peptidyl-transferase center (Englander et al., 2015). That is, if the structure of an amino acid is different from its original form (in this case, it is conjugated with acetate), the ribosome cannot bind to the amino acid. Eventually, ribosome will not be able to use the amino acid for protein elongation. Pyruvate is a precursor for the synthesis of acetate. In the case of NMA, enough or excess availability of acetate might cause feedback inhibition of pyruvate dehydrogenase which is the enzyme responsible for converting pyruvate into acetyl CoA and CO_2, eventually inhibiting the pyruvate pathway (Pietrocola et al., 2015).

Comprehensive Proteomics Analysis for Pathways related to Milk Protein Synthesis

L-Met, CMA, NMA vs Control. A total of 39 upregulated and 77 downregulated proteins, 62 upregulated and 80 downregulated proteins, 50 upregulated and 81 downregulated proteins were observed in L-Met, CMA, and NMA treated groups, respectively (Table 1) using t-test comparing with the control ($P < 0.05$). In addition, several proteins involved in protein and energy metabolism were examined due to the effects of experimental treatments. Eukaryotic Translation Initiation Factor 3 Subunit A (EIF3A) was upregulated by NMA while EIF4A2 (Eukaryotic Translation Initiation Factor 4A2) was upregulated by L-Met. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was upregulated by supplementing both L-Met and CMA. Upregulation of EIF3A by NMA and EIF4A2 by L-Met are important for stimulating protein

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synthesis because these genes are important transcription factors for initiation of protein translation.

Interestingly, GADPH enzyme translation was stimulated upon addition of L-Met and CMA, but not by NMA. This enzyme stimulates the important energy-yielding process of 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). The stimulation of GADPH in L-Met or CMA group, but not in NMA group, suggests that there is enough available energy supplied by acetate in NMA group. Eventually, there will be no need to produce energy via the glycolysis pathway (Nicholls, Li, & Liu, 2012).

Protein pathway analysis revealed that NMA treatment, but not CMA treatment, stimulated ATP synthesis, cell cycle, ubiquitin proteasome, and TGF-β signaling pathways, On the other hand, CMA and L-Met treatments, but not NMA treatment, stimulated PI3 kinase and pyruvate metabolism pathways, indicating that acetate group conjugated in CMA could not be utilized as an energy source in MAC-T cells, consequently resulting in cells to produce energy via pyruvate pathway and PI3 pathway (Table 2).

In aforementioned discussion, pyruvate as a precursor for the synthesis of acetate, additional supply of acetate would cause feedback inhibition of pyruvate dehydrogenase enzyme which is responsible for converting pyruvate into acetyl CoA and CO_2, eventually inhibiting the pyruvate pathway. In case of PI3 pathway, there would be no need to produce energy from other sources like glucose since there is abundant supply of energy source in the form of acetate in NMA treatment group (Table 3). It is well known that PI3 pathway is involved in Insulin Receptor Pathway (INSR) which catalyzes the absorption of glucose from blood to cell (Pessin and Saltiel, 2000).

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CMA, NMA vs L-Met. Using L-Met as the basis for comparison, there were 67 and 37 increased protein for CMA and NMA. For decreased protein, there were 47 and 40 in CMA and NMA and 114 and 77 in total for CMA and NMA groups (Table 4). In case of CMA, there was increased in the protein related to energy synthesis like Glyceraldehyde-3-Phosphate Dehydrogenase (GADPH) and H+ Transporting, Mitochondrial F1 Complex, ATP Synthase, Alpha Subunit 1 (ATP5A1) and also the stimulation of pentose phosphate pathway which are related to energy synthesis. As we mentioned earlier, due to conjugation of acetate to L-Met, the acetate source cannot be utilized for energy source, so the cells must synthesize energy through other pathways. In case of NMA, it stimulated proteins related to protein synthesis like Eukaryotic Translation Initiation Factor 3 Subunit A (EIF3A) and Elongation Factor 1-alpha 1 (EEF1A1) but surprisingly a decreased in Ribosomal ProteinS21 (RPS21) and Ribosomal Protein S12 (RPS21). There was also an increased in glycolysis pathway in proportion to the increased in protein synthesis. One interesting result also is the increased in cholesterol biosynthesis in NMA group due to availability of acetate from unconjugated form of acetate (Table 5).

CMA vs. NMA. Conducting a t-test statistical comparison between CMA and NMA group, it was found that 75 increased, 52 decreased and 127 in total protein which are significantly different between CMA and NMA groups (Table 6). For CMA group, there was increased in proteins related to protein synthesis such as Eukaryotic Translation Initiation Factor 3 Subunit A (EIF3A), Ribosomal Protein S21(RPS21), Eukaryotic Translation Initiation Factor 4A2 (EIF4A2) and Ribosomal Protein S12 (RPS21). But some proteins also related to protein synthesis were decreased in CMA group such as Eukaryotic Translation Initiation Factor 4A1 (EIF4A1) and Elongation factor 1-alpha 1 (EEF1A1). Fatty acid synthase (FASN),

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a kind of multi-enzyme protein which catalyzes fatty acid synthesis, was lower in CMA compared to NMA that may imply a larger supply of acetate in NMA than CMA group (Table 7).

Comprehensive Metabolome 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 forms of L-Met, CMA group has a sharp decrease in terms of intracellular Met level compared to NMA group ($P < 0.05$). This could mean that the conjugated form of L-Met has low absorption rate into the cell probably due to its larger size. In terms of other amino acids, serine, glutamine and aspartate have higher absorption rate in CMA group while valine, isoleucine, leucine, tyrosine and phenylalanine have higher absorption rate in NMA group. The logical explanation for this is the competition of free amino acids for amino acid transporters. As we know, different amino acids have their own specific transporters. In case of serine and methionine, they share the same amino acid transporter ATA2 (Reimer, Chaundhry, Gray, & Edwards, 2000; Sugawara et al., 2000a; Yao et al., 2000) and for methionine and glutamine, they shared bo,+ (Chairoungdua et al., 1999) and B0,+ (Sloan & Mager, 1999) amino acid transport systems. So the decrease in L-Met absorption rate in L-Met in CMA caused lesser competition upon absorption of serine and glutamine that caused their higher absorption rate.

L-Met, CMA, NMA vs Control. Table 8 displays the list of pathway detection metabolites. Most of them are involved in the metabolism of energy and amino acids. Such results are expected since treatments are mainly about addition of amino acid and energy sources in the present study. Based on metabolite pathway analysis, CMA supplementation, but not NMA, stimulated Ala-Asp-Glu, Aminoacyl-tRNA biosynthesis, and glyoxylate and

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dicarboxylate metabolism pathway. However, NMA, but not CMA, stimulated inositol phosphate metabolism and pentose phosphate metabolic pathway.

Results of metabolite analysis also showed that CMA treatment caused increases of 12 metabolites and decrease of UMP ($P < 0.05$) (Table 9). On the other hand, NMA treatment caused increases of 13 metabolites and decreases of IMP and pantothenate ($P < 0.05$). Addition of different forms of methionine 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 intermediates of Krebs Cycle. On the other hand, CMA supplementation caused increases of energy metabolite NADH, malate, and xylulose-5-phosphate, an intermediate of pentose phosphate pathway. In the case of NMA supplementation, glucose intermediates (such as glucose-6-phosphate, fructose-6-phosphate, and glucose-1-phosphate) and pentose phosphate pathway intermediates (xylulose-5-phosphate and N-acetyl-L-glutamate) were increased. Previous researches have shown that energy intermediates could stimulate protein synthesis indirectly by inhibiting AMPK signal (Sarbassov, Ali, & Sabatini, 2005; Appuhamy et al., 2012), 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 & Ruderman, 2009). AMPK may modulate the decrease in energy status, consequently inhibiting very high energy consuming protein synthetic process (Proud, 2007).

CMA, NMA vs L-Met. Looking at the detected metabolite, there was an interesting decreased in Met and acetate-glutamate level inside the cell which means that CMA might not be properly absorbed due to their larger conjugated size (Table 10). On the other hand, in terms of NMA, there was an increased in Met as well as other amino acids like valine, isoleucine,

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leucine, tyrosine and phenylalanine. The increased in Met and acetate-glutamate level in NMA and not in CMA may imply that Met as well as acetate-glutamate are readily available or properly absorbed inside the cell via the plasma membrane amino acid transport systems in their unconjugated form. The decreased in pyruvate metabolite in NMA could mean an already abundant amount of acetate for energy utilization.

CMA vs. NMA. Again, looking at the detected metabolite data, methionine as well as the acetate-glutamate level was lower in CMA than NMA group (Table 11). As we have mentioned this may be due to larger size of CMA due to its conjugated state and cannot be readily absorbed inside the cell. Also there were observable increased of metabolites related to energy synthesis such as Glycerol-3-Phosphate, NAD^+, Dihydroxyacetone phosphate, Glycerate-3-Phosphate, Fructose 1,6-bisphosphatase, Citrate, NADP, Phosphoenol pyruvate, NADH and NADPH. This may be due to lesser supply of acetate in CMA than NMA group. The acetate in NMA group is in free unconjugated form so it can be readily absorbed and utilized by the cell.

To summarize interactions of the addition of different forms 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 glycolysis, pyruvate metabolism, and ATP synthesis pathways. Metabolites such as pyruvate, malate, and fumarate were also stimulated. They are involved in energy production and metabolism (Figure 10). This stimulation promoted the stimulation of mTOR activity that elevated *β-casein* expression, eventually increasing protein synthesis.

On the other hand, the addition of CMA stimulated PI3 Kinase and GAPDH enzymes, EIF4A2 protein, glycolysis, pentose phosphate and pyruvate metabolism pathways, and

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xyluose-5-phosphate, NADH, and malate metabolites (Figure 11), all of which promoted *mTOR* and *β-casein* gene expression, thus increasing protein synthesis.

Lastly, addition of NMA stimulated EIF3A protein, glycolysis, pentose phosphate, and ATP synthesis pathways as well glucose-6-phosphate, fructose-6-phophate, xylulose-5-phosphate, glucose-1-phosphate, and N-acetyl-L-glutamate (Figure 12), all of which increased *β-casein* gene expression that finally increased protein synthesis. One interesting difference that we can observe between the addition of conjugated form of CMA and non-conjugated form NMA is that pyruvate metabolism and PI3 kinase are activated in CMA, but not in NMA. When pyruvate metabolism and PI3 kinase are activated, cells will produce energy from acetate (in case of pyruvate metabolism) and increase glucose utilization (in case of PI3 which involves insulin signaling). This concludes that the conjugated form of L-Met and acetate (CMA) cannot utilize energy from acetate *in vitro*. However, situation will be different *in vivo* because animals already have enzymes in the intestine and liver that can cleave the conjugated form into its unconjugated form.

CONCLUSION

Results of this study showed that NMA-treated group exhibited much better performance in stimulating the expression of *β-casein* mRNA. It 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, NMA-treated group resulted in higher protein concentration in MAC-T cells compared to other groups in terms of protein inside MAC-T cells and in the culture medium ($P < 0.05$). This outcome may suggest that CMA can be effectively utilized to increase protein synthesis in bovine mammary epithelial cells, especially if they are separated into digested form of NMA, because the

conjugated form of L-Met and acetate (CMA) does not have higher bioavailability for synthesis of milk protein in mammary gland.

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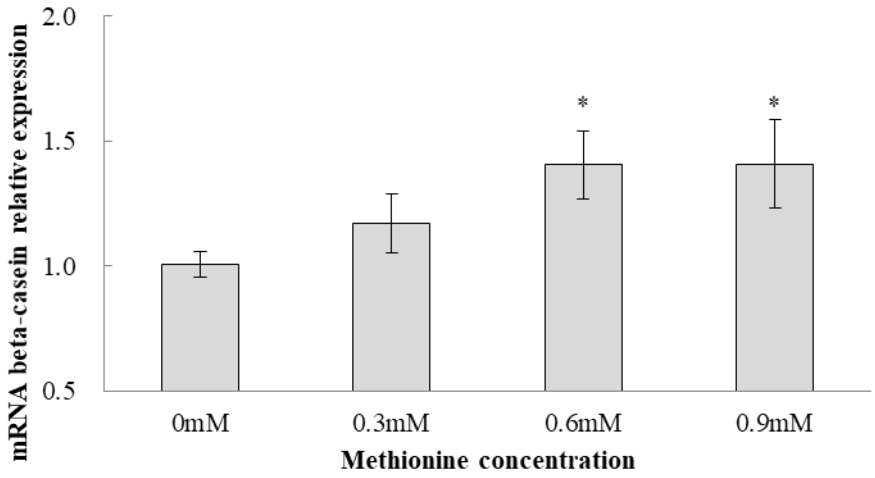

Figure 1. *β-casein* mRNA expression level in MAC-T cells incubated with different levels of L-methionine (0, 0.3, 0.6, 0.9 mM) for 24 h. Values are expressed as means ± SE (n = 6 per group). Letters * indicate significant differences at $P < 0.05$ by student t-test comparing control (0 mM)

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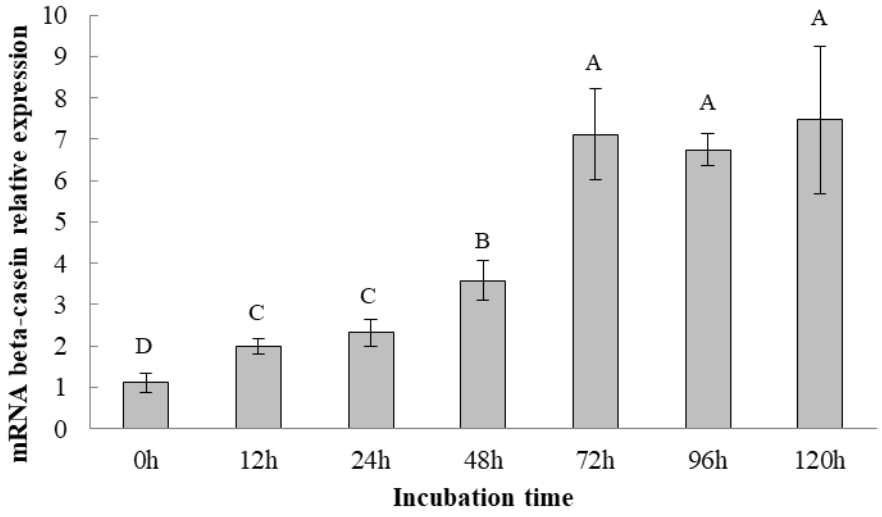

Figure 2. *β-casein* mRNA expression level in MAC-T cells incubated with 0 mM L-methionine at different time points (0, 24, 48, 72, 96, 120 h). Values are expressed as means ± SE (n = 6 per group) and A, B, C, D indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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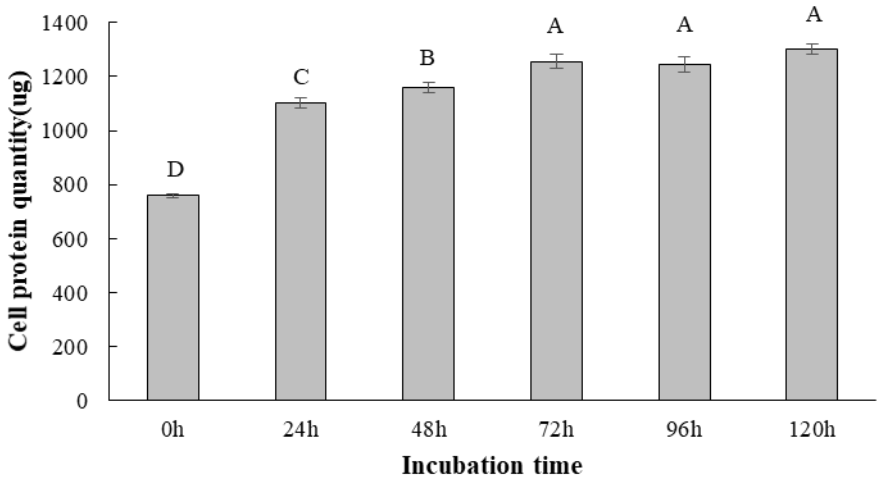

Figure 3. Cell protein quantity in MAC-T cells incubated with 0 mM L-methionine at different time points (0, 24, 48, 72, 96, 120 h). Values are expressed as means ± SE (n = 6 per group) and A, B, C indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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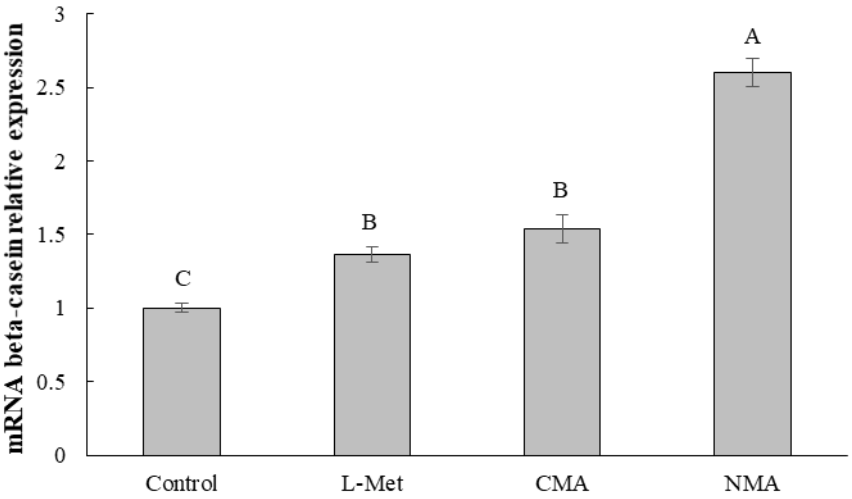

Figure 4. . *β-casein* mRNA expression level in MAC-T cells incubated with control (0 mM), L-Met (0.6 mM), CMA (0.6 mM) and NMA (0.6 mM). Values are expressed as means ± SE (n = 6 per group) and A, B, C indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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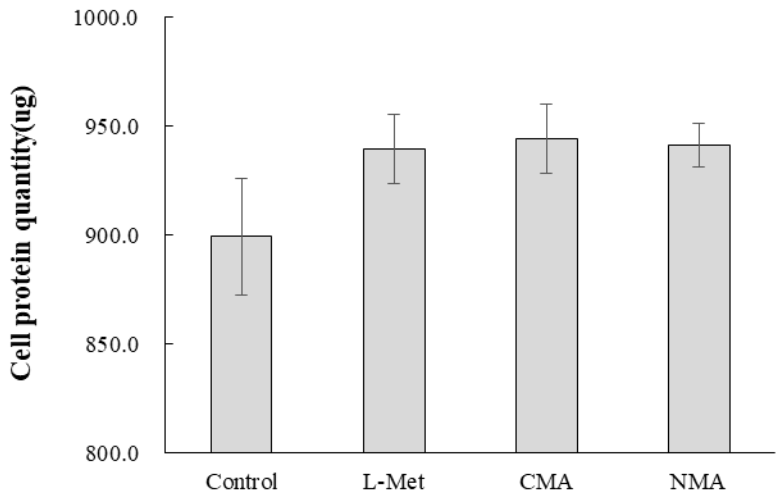

Figure 5. Cell protein quantity in MAC-T cells incubated with control (0 mM), L-methionine (0.6 mM), CMA (0.6 mM) and NMA (0.6 mM). Values are expressed as means ± SE (n = 6 per group).

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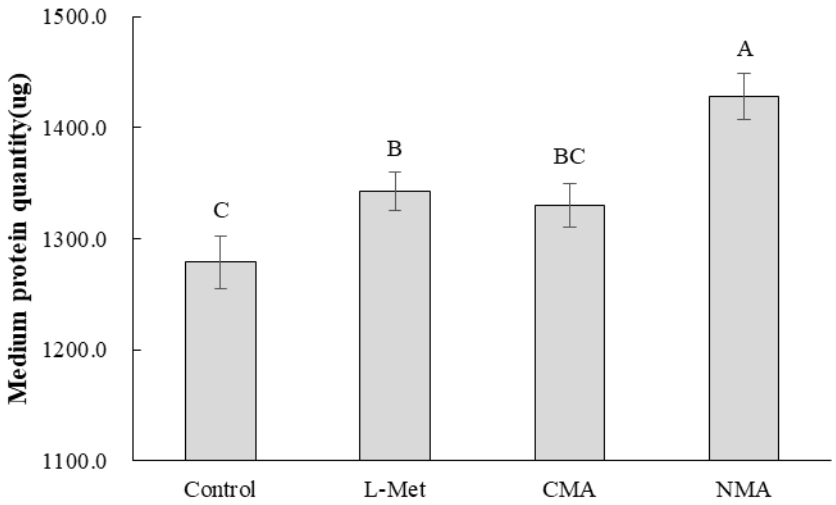

Figure 6. Medium protein quantity in MAC-T cells incubated with control (0 mM), L-methionine (0.6 mM), CMA (0.6 mM) and NMA (0.6 mM). Values are expressed as means ± SE (n = 6 per group) and A, B, C indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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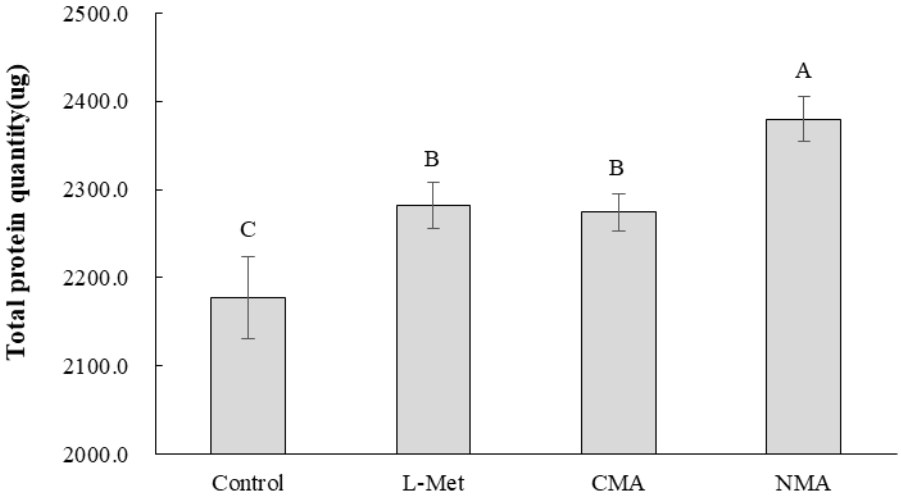

Figure 7. Total protein quantity (cell and medium) in MAC-T cells incubated with control (0 mM), L-methionine (0.6 mM), CMA (0.6 mM) and NMA (0.6 mM). Values are expressed as means ± SE (n = 6 per group) and A, B, C indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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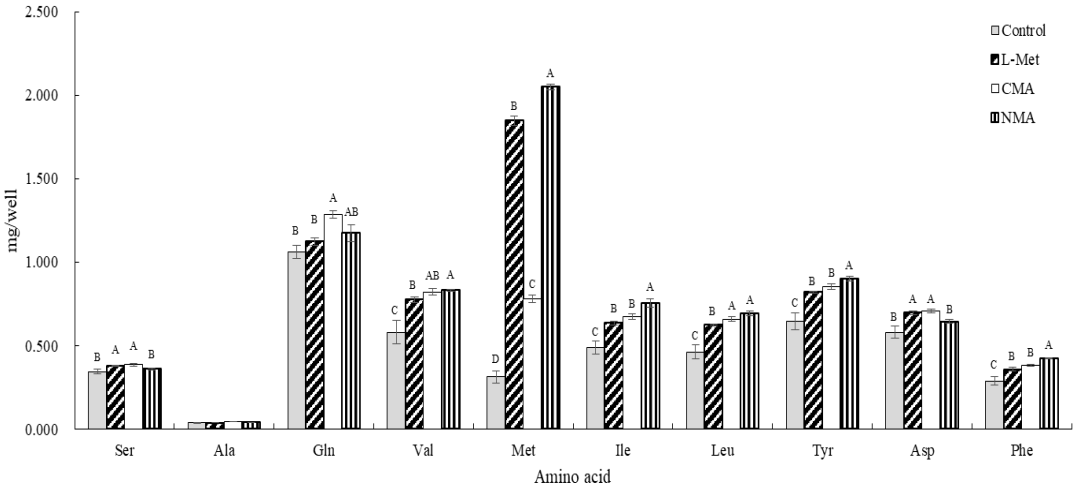

Figure 8. Amino acids in MAC-T cells incubated with control (0 mM), L-methionine (0.6 mM), CMA (0.6 mM) and NMA (0.6 mM). Values are expressed as means ± SE (n = 3 per group) and A, B, C, D indicate significant differences at $P < 0.05$ by Duncan Multiple Range Test.

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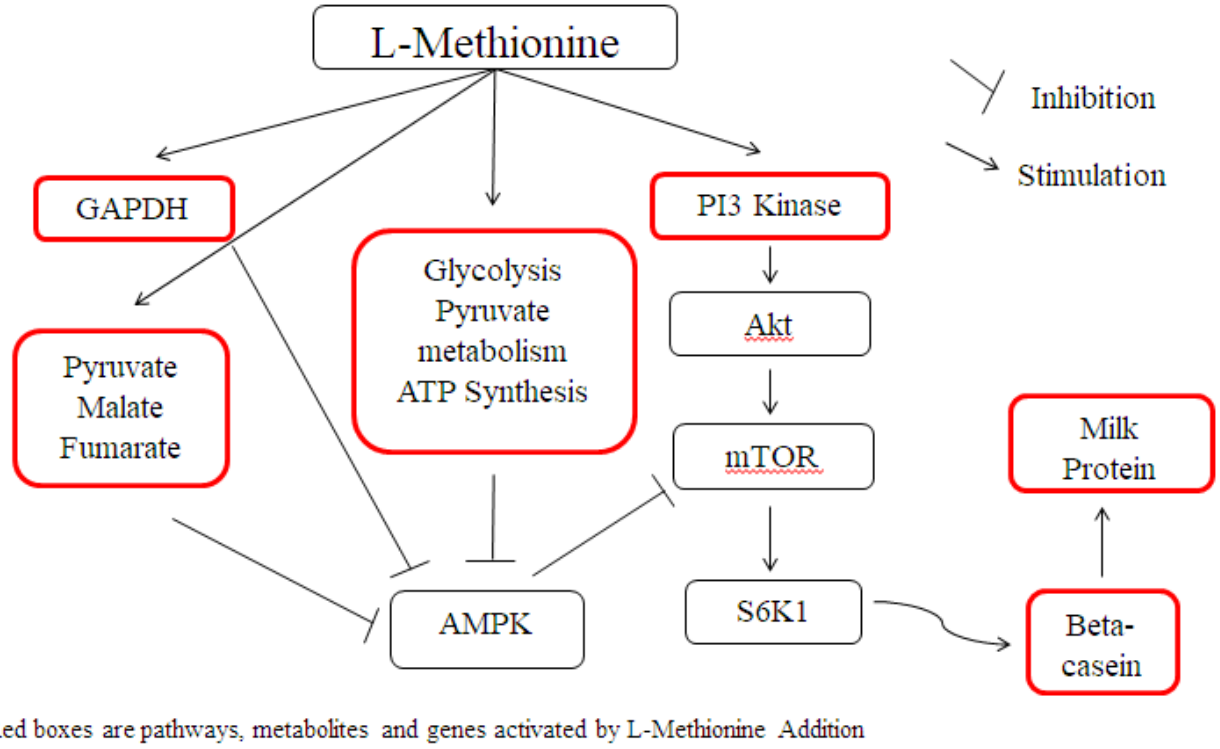

Figure 9. Diagram showing the effect of L-methionine on protein synthesis pathway.

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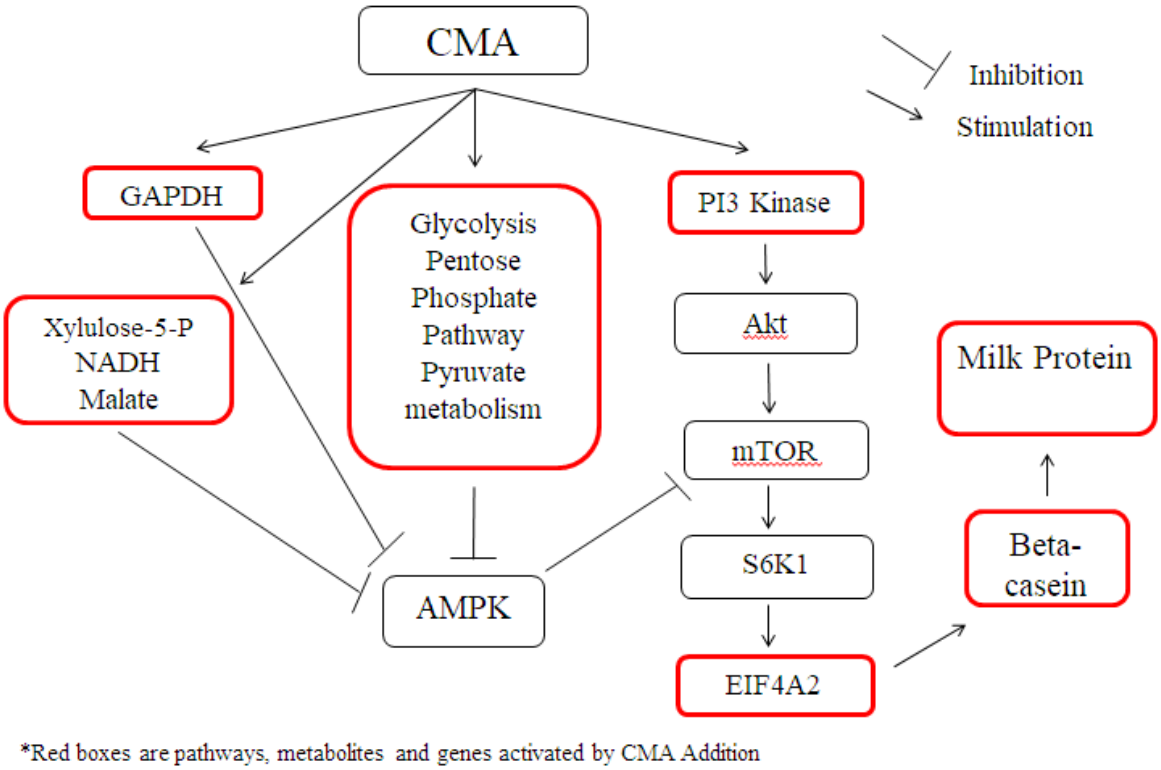

Figure 10. Diagram showing the effect of CMA on protein synthesis pathway.

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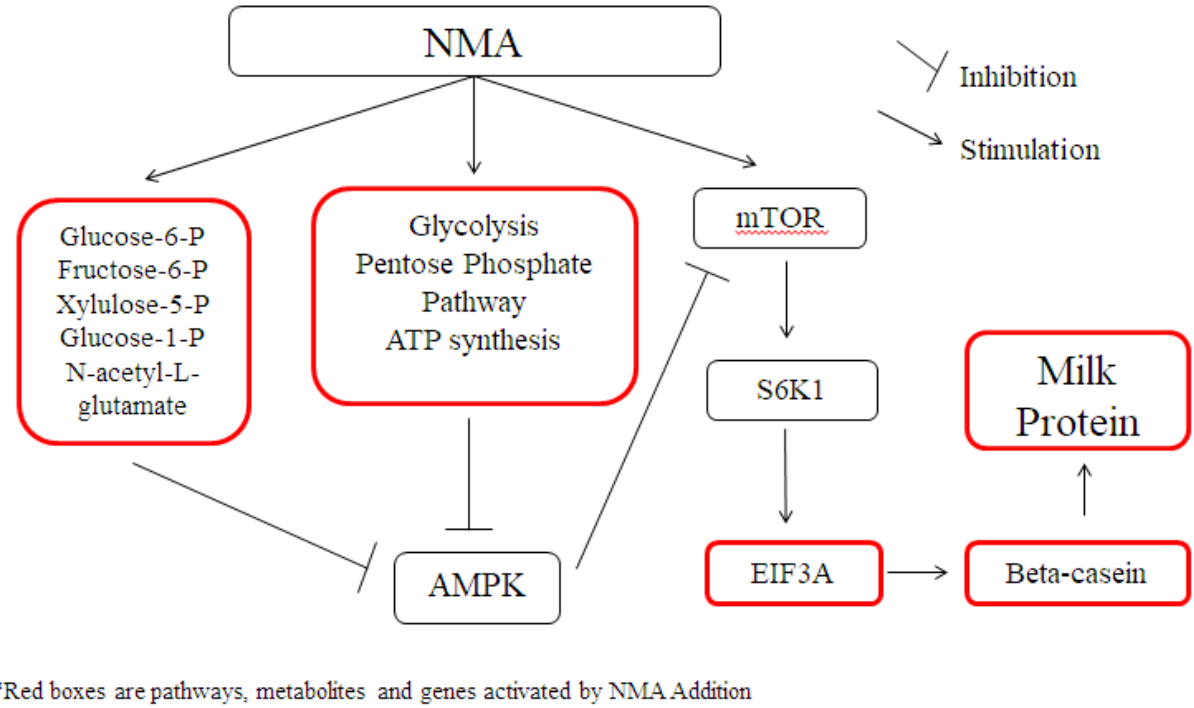

Figure 11. Diagram showing the effect of NMA on protein synthesis pathway.

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

Detection Protein	**L-Met[1]**	**CMA[2]**	**NMA[3]**
Increasing Number	39	62	50
Decreasing Number	77	80	81
Total Protein Number	116	142	131
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]CMA = conjugated L-methionine and acetate

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[3]NMA = non-conjugated L-methionine and acetate.

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

Detected Pathway	L-Met[1]	CMA[2]	NMA[3]
Apoptosis signaling		●	
ATP synthesis	●		●
Cell cycle			●
Dopamine receptor mediated signaling pathway		●	
FAS signaling		●	●
Glycolysis		●	●
Hypoxia response via HIF activation		●	
Nicotine pharmacodynamics		●	
Oxidative stress response		●	
Pentose phosphate		●	
PI3 kinase	●	●	
Purine metabolism		●	
Pyruvate metabolism	●	●	
Ubiquitin proteasome			●

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TGF- β signaling			●

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

[1]L-Met = L-methionine

[2]CMA = conjugated L-methionine and acetate

[3]NMA = non-conjugated L-methionine and acetate.

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

Detected Pathway	L-Met[1]	CMA[2]	NMA[3]
5HT2 type receptor mediated signaling	●	●	●
Alzheimer's disease-presenilin	●	●	●
Apoptosis signaling		●	
ATP synthesis	●		●
Cadherin signaling	●	●	●
CCKR signaling map	●	●	●
Cell cycle			●
Cholesterol biosynthesis	●	●	●
Cytoskeletal regulation by Rho GTPase	●	●	●
De novo purine biosynthesis	●	●	●
De novo pyrimidine deoxyribonucleotide biosynthesis	●	●	●
De novo pyrimidine ribonucleotides biosynthesis	●	●	●
Dopamine receptor mediated signaling pathway		●	
EGF receptor signaling	●	●	●

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FAS signaling		●	●
FGF signaling	●	●	●
Fructose galactose metabolism			
Glycolysis		●	●
Gonadotropin-releasing hormone receptor	●	●	●
HeterotrimericG-protein signaling pathway -Gi alpha and Gs alpha mediated	●	●	●
Heterotrimeric G-protein signaling pathway -Gq alpha and Go alpha mediated	●	●	●
Histamine H1 receptor mediated signaling	●	●	●
Huntington's disease	●	●	●
Hypoxia response via HIF activation		●	
Inflammation mediated by chemokine and cytokine signaling	●	●	●
Integrin signaling	●	●	●
Nicotine pharmacodynamics		●	
Nicotinic acetylcholine receptor signaling	●	●	●
Oxidative stress response		●	
Oxytocin receptor mediated signaling	●	●	●
p53	●	●	●
Parkinson's disease	●	●	●
Pentose phosphate		●	
PI3 kinase	●	●	

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Purine metabolism		●	
Pyruvate metabolism	●	●	
Salvage pyrimidine ribonucleotides	●	●	●
Thyrotropin-releasing hormone receptor signaling	●	●	●
Wnt signaling	●	●	●
Ubiquitin proteasome			●
TGF- β signaling			●

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

[1]L-Met = L-methionine

[2]CMA = conjugated L-methionine and acetate

[3]NMA = non-conjugated L-methionine and acetate.

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Table 4. List of differently expressed proteins in MAC-T Cells (in comparison to L-Met)

Detection Protein	**CMA[1]**	**NMA[2]**
Increasing Number	67	37
Decreasing Number	47	40
Total Protein Number	114	77
List of Selected Downregulated and Upregulated Proteins		
Eukaryotic Translation Initiation Factor 3 Subunit A (EIF3A)		▲
Ribosomal Protein S21(RPS21)		▼
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	▲	▼
Eukaryotic Translation Initiation Factor 4A1 (EIF4A1)	▼	
EIF4A2 (Eukaryotic Translation Initiation Factor 4A2)	▲	
ATP Synthase, H+ Transporting, Mitochondrial F1 Complex, Alpha Subunit 1 (ATP5A1)	▲	▲
Ribosomal Protein S12 (RPS21)		▼
Elongation factor 1-alpha 1 (EEF1A1)		▲

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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]CMA = conjugated L-methionine and acetate

[2]NMA = non-conjugated L-methionine and acetate.

Table 5. List of all protein pathways affected by treatment supplementation (L-Met as control).

Detected Pathway	CMA[1]	NMA[2]	Both
5HT2 type receptor mediated signaling pathway		●	
ALP23B_signaling_pathway		●	
Apoptosis signaling pathway	●	●	
Alzheimer disease-presenilin pathway		●	
ATP synthesis	●	●	
BMP_signaling_pathway-drosophila		●	
Cadherin signaling pathway	●	●	
Cholesterol biosynthesis		●	
Cytoskeletal regulation by Rho GTPase	●	●	
De novo purine biosynthesis	●		
De novo pyrimidine deoxyribonucleotide biosynthesis		●	
De novo pyrmidine ribonucleotides biosynthesis		●	
EGF receptor signaling pathway	●	●	●
FAS signaling pathway	●		

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FGF signaling pathway		●	●
Glycolysis		●	
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	●		
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	●		
Histamine H1 receptor mediated signaling pathway		●	
Huntington disease	●	●	
Hypoxia response via HIF activation			
Inflammation mediated by chemokine and cytokine signaling pathway	●	●	
Integrin signaling pathway	●	●	●
MYO signaling pathway		●	
Nicotinic acetylcholine receptor signaling pathway	●	●	
Nicotine pharmacodynamics pathway	●		
Oxytocin receptor mediated signaling pathway		●	
p53 pathway	●	●	●
Parkinson disease	●	●	●
Pentose phosphate pathway	●		
TGF-β signaling pathway		●	
Thyrotropin-releasing hormone receptor signaling pathway		●	
Wnt signaling pathway		●	

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

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[1]CMA = conjugated L-methionine and acetate

[2]NMA = non-conjugated L-methionine and acetate.

Table 6. List of differently expressed proteins in MAC-T Cells (in comparison to NMA[2])

Detection Protein	**CMA[1]**
Increasing Number	75
Decreasing Number	52
Total Protein Number	127
List of Selected Downregulated and Upregulated Proteins	
Eukaryotic Translation Initiation Factor 3 Subunit A (EIF3A)	▲
Ribosomal Protein S21(RPS21)	▲
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	▲
Eukaryotic Translation Initiation Factor 4A1 (EIF4A1)	▼
Eukaryotic Translation Initiation Factor 4A2 (EIF4A2)	▲
Ribosomal Protein S12 (RPS21)	▲
Elongation factor 1-alpha 1 (EEF1A1)	▼

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ATP synthase subunit β, mitochondrial (ATP5B)	▼
FASN Fatty acid synthase	▼

Selection Criteria: Upregulated, > 2-fold in protein expression vs. NMA,
Downregulated: < 0.5-fold in protein expression vs. NMA.
[1]CMA = conjugated L-methionine and acetate
[2]NMA = non-conjugated L-methionine and acetate.

Table 7. List of all protein pathways affected by treatment supplementation (NMA[2] as control).

Detected Pathway	CMA[1]
Apoptosis signaling pathway	●
Huntington disease	●
Integrin signaling pathway	●
De novo pyrimidine deoxyribonucleotide biosynthesis	●
Parkinson disease	●
FAS signaling pathway	●
Alzheimer disease-presenilin pathway	●
Nicotine pharmacodynamics pathway	●
PI3 kinase pathway	●
Cytoskeletal regulation by Rho GTPase	●
Nicotinic acetylcholine receptor signaling pathway	●
De novo purine biosynthesis	●
Pentose phosphate pathway	●
Inflammation mediated by chemokine and cytokine signaling	●

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pathway	
Wnt signaling pathway	●
EGF receptor signaling pathway	●
Cadherin signaling pathway	●
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	●
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway 2	●
Salvage pyrimidine ribonucleotides	●
De novo pyrimidine ribonucleotides biosynthesis	●

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

[1]CMA = conjugated L-methionine and acetate

[2]NMA = non-conjugated L-methionine and acetate.

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Table 8. List of Pathway Related to Detection Metabolite

Metabolite related pathway	L-Met[1]	CMA[2]	NMA[3]
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			●

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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]CMA = conjugated L-methionine and acetate

[3]NMA = non-conjugated L-methionine and acetate.

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Table 9. List of Detected Metabolite affected by application of treatment

Category	L-Met[1]	CMA[2]	NMA[3]
Increasing Metabolite List	Pyruvate	NADH	Phenylalanine
	2-Oxoglutarate	Valine	Glucose 6-phosphate
	Fumarate	Aspartate	Fructose 6-phosphate
	N-acetyl-L-glutamate	Phenylalanine	Xylulose5-P
	Malate	Xylulose5-P	2-oxoisovalerate
	Aspartate	2-oxoisovalerate	Malate
	Met	Malate	Met
	Leu	Met	Leu
	Tyrosine	Leu	Tyrosine
	Ile	Tyrosine	N-acetyl-L-glutamate
		6-Phosphogluconate	Glucose 1-phosphate
		Ile	6-Phosphogluconate
			Ile
Decreasing Metabolite List		UMP	IMP
			Pantothenate

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

[1]L-Met = L-methionine

[2]CMA = conjugated L-methionine and acetate

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[3]NMA = non-conjugated L-methionine and acetate.

Table 10. List of Detected Metabolite using L-Met as control.

Category	CMA[1]	NMA[2]
Increasing Metabolite List	Alanine	Valine
	Glutamine	Methionine
	2-oxoisovalerate	Isoleucine
	Fructose 1,6-bisphosphatase	Leucine
	NADH	Tyrosine
	FAD	Phenylalanine
		Glucose-6-Phosphate
		Fructose-6-Phosphate
		Fructose-1-Phosphate
		2-oxoisovalerate
		Acetate-Glutamate
Decreasing Metabolite List	Methionine	Aspartate
	UMP	Glycerol-3-Phosphate
	IMP	Pyruvate
	AMP	NAD+
	Acetate-Glutamate	UMP
	Fumarate	IMP
	Phosphoenolpyruvate	GMP
	UDP-glucuronic acid	Pantothenate
		AMP

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		6-Phosphoglycerate
		Citrate
		Guanosine diphosphate
		NADPH

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

[1]CMA = conjugated L-methionine and acetate

[2]NMA = non-conjugated L-methionine and acetate.

Table 11. List of Detected Metabolite (NMA[2] as control).

Category	**CMA[1]**	**p-value**
Decrease Metabolite List	Methionine	<0.0001
	Isoleucine	0.046
	Phenylalanine	0.002
	Glucose-6-Phosphate	0.002
	Fructose-6-Phosphate	0.001
	Glucose-1-Phosphate	0.008
	Acetate-Glutamate	<0.0001
	6-Phosphoglycerate	0.012
Increase Metabolite List	Serine	0.046
	Aspartate	0.020
	Glycerol-3-Phosphate	0.044
	NAD^+	0.007
	Dihydroxyacetone phosphate	0.033
	Inosinic acid	0.002
	Pantothenate	0.042
	Glycerate-3-Phosphate	0.012
	Fructose 1,6-bisphosphatase	0.022
	Citrate	0.029
	NADP	0.048
	Guanosine diphosphate	0.062

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	Phosphoenol pyruvate	0.085
	NADH	0.024
	UDp-glucoronic acid	0.038
	NADPH	0.046

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

[1]CMA = conjugated L-methionine and acetate

[2]NMA = non-conjugated L-methionine and acetate.