

Article

Supplementing L-isoleucine increases medium protein and alters the expression of genes and proteins involved in milk protein synthesis and energy metabolism in bovine mammary cells

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Abstract: The objective of this study was to determine the effects of supplementing L-isoleucine (L-Ile) on milk protein synthesis, using an immortalized bovine mammary epithelial (MAC-T) cell line. In this case, the cells were treated with 0, 0.3, 0.6, 0.9, 1.2 and 1.5 mM of supplemental Isoleucine (Ile), and the most efficient time for protein synthesis for each amino acid was determined by measuring the cell, medium and total protein at 0, 24, 48, 72 and 96 h. Confirmatory tests showed that 48h incubation time and 0.6 mM dosage of L-Ile are considered as the optimal time and dosage. The mechanism of milk protein synthesis was elucidated through proteomics analysis to clarify the metabolic pathway. When the L-Ile was supplemented, extracellular protein (medium protein) reached a peak at 48h, whereas in the case of the intracellular cell protein, it was shown to have reached to its peak at 24h in all L-Ile dosage treatments. In total, it is noted that there were 63 upregulated and 52 downregulated proteins. The results of the protein pathway analysis showed that the L-Ile group stimulated insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade, insulin/IGF pathway-protein kinase B signaling cascade, p53 pathway, de novo purine biosynthesis, Wnt signaling pathway, glycolysis, pentose phosphate pathway, and ATP synthesis which are pathways involved and related to protein and energy metabolism. Together, these results demonstrate that L-Ile supplementation was effective in stimulating β -casein synthesis by stimulating genes and pathways which are significantly related to protein and energy metabolism.

Keywords: beta casein; MAC-T cells; Ile; milk protein synthesis; omics; proteomics

1. Introduction

In this sense, it is noted that among these nutrients, amino acids (AAs) play a very crucial role [1]. By the same token, AAs mainly act as building blocks for protein synthesis [2]. These amino acids do not only serve as substrates for protein synthesis, but also serve as signaling regulatory molecules [3]. In this case, the AAs not only function as precursors for protein synthesis, but also function in regulating protein synthesis rates [4]. Evidently, some AAs have been proven to affect translation initiation and elongation rates via 2 major pathways: the integrated stress response (ISR) and the mammalian target of rapamycin (mTOR) pathways [5]. The ISR regulates methionyl- transfer RNA recruitment to the 40S ribosomal subunit by the presence of eukaryotic initiation factor (eIF) 2 [5].

Generally speaking, it is reported that Isoleucine (Ile) may function in addition to Leucine (Leu) to stimulate milk protein synthesis [6]. The supplementation of Ile which is ineffective in stimulating synthesis of muscle protein [7] may minimize partitioning of AAs to muscle towards milk protein

synthesis. The instance of Ile supplementation has been shown to increase milk protein synthesis in lactating sows, while the supplementation of a mixture of the branched-chain amino acids (BCAAs) had no effect [7]. At the same time, in mammary tissue, Ile or Leu supplementation alone increased casein fractional synthesis rate (CFSR) [8,9].

The mTOR, a protein kinase present in the rapamycin-sensitive mTOR complex 1 (mTORC1) [10] partly mediates the stimulation of protein synthesis by AAs (Kim, 2009). The mTOR pathway centers around mTORC1 [12]. From here, the mTOR will phosphorylate the downstream proteins regulating the rate of translation initiation and elongation (Dunlop and Tee, 2009). The provision of Ile alone also increased mTOR phosphorylation [8,9]. Notably, the removal of L-Ile or all EAA reduced mTOR and ribosomal protein S6 (rpS6) phosphorylation in MAC-T cells [9]. It is shown that supplementation of L-Ile also increased S6K1 and rpS6 phosphorylation [13]. Consequently, the L-Ile linearly increased rpS6 phosphorylation [14]. Unlike liver and skeletal muscle, the significance of Ile as signaling AA in the mammary glands was indeed proven in this case [15]. It is noted that the fractional synthesis rate (FSR) declined when L-Ile was removed in the media and was positively correlated with mTOR phosphorylation, and negatively correlated with eIF2a phosphorylation [9].

A recent study compared the effect of acetate-protected L-methionine for milk protein synthesis in MAC-T cell line [16]. In this study, different treatments upregulate and downregulate several proteins and metabolic pathways involved in the process of energy and protein metabolism. However, to the best of our knowledge, no studies yet have been performed or completed using omics (transcriptomics/proteomics) with approaches to analyze the effects of supplementing L-Ile on milk protein (β -casein) synthesis. The objective of the present study was to investigate the effect of supplementing L-Ile on stimulation of milk protein synthesis-linked pathways to increase β -casein synthesis in MAC-T cells, and to elucidate the metabolic pathway involved in β -casein synthesis using omics (transcriptomics and proteomics) data.

2. Materials and Methods

2.1 AA Dosage and Sampling Time

In a preliminary study to determine the optimum Ile dosage and sampling time, MAC-T cells obtained from the University of Vermont, Burlington, VT, USA, were seeded into 10-cm dishes (TPP, Trasadingen, Switzerland), and cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) basic medium (Thermo Fisher Scientific, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin (Thermo Fisher), 5 μ g/mL insulin, 1 μ g/mL hydrocortisone, and 50 μ g/mL gentamycin (Sigma-Aldrich Corp., St. Louis, MO, USA) at 37°C in a 5% CO₂ incubator [17,18]. The cells were then seeded into six-well multi-well plates (BD Falcon, Franklin Lakes, NJ, USA) and incubated with basic growth medium.

In this context, when MAC-T cells reached about 90% confluence, DMEM/F12 basic medium was replaced with lactogenic DMEM/F12 differentiation medium without FBS to differentiate MAC-T cells into β -casein-secreting cells. It is noted that this medium contained 5 μ g/mL bovine insulin, 1 μ g/mL hydrocortisone, 100 U/mL penicillin/streptomycin, 50 μ g/mL gentamycin, and 5 μ g/mL prolactin (Sigma-Aldrich) [17,18]. In this case, the AA profile of the differentiation medium was as follows: 0.70 mM Arg, 0.10 mM Cys, 2.5 mM Gln, 0.25 mM Gly, 0.15 mM His, 0.42 mM Ile, 0.45 mM Leu, 0.50 mM Lys, 0.12 mM Met, 0.22 mM Phe, 0.25 mM Ser, 0.45 mM Thr, 0.04 mM Trp, 0.21 mM Tyr, and 0.45 mM Val.

In this context, the cells were treated with 0, 0.3, 0.6, 0.9, 1.2 and 1.5 mM of supplemental Ile and the most efficient time for protein synthesis for each amino acid was determined by measuring cell, medium and total protein at 0, 24, 48, 72 and 96 h. Then, a confirmatory study was performed to

determine the ideal dosage of Ile on its optimal cultivation time. In all experiments, each treatment was replicated six times, with treatments randomly assigned among the wells of a single 6-well plate.

2.2 RNA Extraction and cDNA Synthesis

The total RNA was extracted from the MAC-T cells using TRIzol (Life Technologies Corporation, Carlsbad, CA, USA). At that time, the RNA quality and quantity were evaluated using a NanoDrop 1000 spectrophotometer with an RNA-40 module (Thermo Fisher). A_{260/280} values > 1.8 and RIN value >8.0 were considered acceptable using Agilent BioAnalyzer 2100™. Additionally, the cDNA was synthesized using the iScript® cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Directly after incubation at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min, the cDNA was quantified using the ssDNA-33 module of the Thermo NanoDrop 1000 spectrophotometer.

2.3 Real-Time Polymerase Chain Reaction (RT-PCR)

It is emphasized that the RT-PCR was performed using a total volume of 20 µL/well in 96-well micro-well plates (Sigma-Aldrich) and a T100 Thermal Cycler System (Bio-Rad). 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), and 6.3 µL diethyl pyrocarbonate (DEPC)-treated water. At this time, the validated RT-PCR oligonucleotide sequences of forward and reverse primers specific for the target genes were as follows: β-casein forward, 5'-AAATCTGCACCTTCCTCTGC-3'; β-casein reverse, 5'-GAACAGGCAGGACTTTGGAC-3'; mTOR forward, 5'- ATGCTGTCCTGGTCCTTAT-3'; mTOR reverse, 5'- GGGTCAGAGAGTGGCCTTCA-3'; S6K1 forward, 5'-GGACATGGCAGGGGTGTTT-3'; S6K1 reverse, 5'- GGTATTTGCTCCTGTACTT-3'; RPS6 forward, 5'- TGAAGCAGGGTGTCTTGACC-3'; RPS6 reverse, 5'- TCCAGTCCTCCTTGGTCTGT-3'; LDH-B forward, 5'- GCTGCCACGGATGGATTTTG-3'; LDH-B reverse, 5'-AGAGAAACGCCTGCCACATT-3'; β-actin forward, 5'-GCATGGAATCCTGCGGC-3'; and β-actin reverse, 5'-GTAGAGGTCCTTGCGGATGT-3'. RT-PCR reactions were performed by initial incubation at 95°C for 3 min, followed by 50 cycles of denaturation at 95°C for 10 s, annealing at specific temperatures for each primer for 15 s, and extension at 72°C for 30 s. The RT-PCR analysis of gene expression changes relative to the control group was performed using the threshold cycle (2- $\Delta\Delta$ CT) method [19].

2.4 Protein Extraction and Quantification

Following incubation in the treatment medium for 72 h, the culture medium was separated from adherent cells. Then the culture medium was centrifuged at 300 × g for 5 min at 4°C to remove any remaining dead cell fragments. The medium supernatant was transferred to a new tube for protein quantification using a bicinchoninic acid assay (BCA). In the case of adherent cells, the cells were washed twice with ice-cold 1× phosphate-buffered saline (PBS); 200 µL cell lysis buffer (pH 8.3) containing 10 mM Tris/HCl, 8 M urea, 5 mM ethylenediamine tetraacetic acid (EDTA), 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and 1× protease inhibitor cocktail (GE Healthcare, Piscataway, NJ, USA) was added. Similarly, the cell lysates were incubated for 30 min at 20°C and centrifuged at 14,000 rpm for 30 min at 20°C (Wang et al., 2014). Next, the cells were then transferred to a new tube for protein quantification by BCA using the Pierce Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instructions.

2.5 Proteome Analysis

Upon completion of the cell culture, another batch of cellular proteins was extracted using a cell lysis buffer containing 20 mM Tris, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.1% sodium dodecyl sulfate (SDS) and complete EDTA-free Protease Inhibitor Cocktail (Roche, Basel,

Switzerland) after washing twice with ice-cold 1× PBS. Next, the cell lysates were incubated at 4°C for 30 min and centrifuged at 13,000 × g for 10 min at 4°C [20].

For proteome analysis, 100 µg cell lysate proteins were resuspended in 0.1% SDS in 50 mM triethyl ammonium bicarbonate (TEABC), pH 8.0. Next, the proteins were chemically denatured using 10 mM Tris (2-carboxyethyl) phosphine (TCEP) at 60°C for 30 min and alkylated with 50 mM methyl methanethiosulfonate (MMTS) at room temperature for 30 min in the dark. At that time, the proteolytic digestion was conducted using trypsin (protein:trypsin = 50:1, g/g) overnight at 37°C. Digested peptides were desalted and concentrated and then subjected to liquid chromatography (LC)–tandem mass spectrometry (MS/MS) analysis. Likewise, the total peptides were analyzed by the use of a nano ultra-performance LC–MS/electrospray ionization quadrupole time-of-flight (UPLC–MS/ESI–Q–TOF) (Waters, Manchester, UK). Hence, the LC peptide separation was performed using the nano Acquity system equipped with a Symmetry C18 5 µm, 5 mm × 300 µm pre-column and CSH C18 1.7 µm, 25 cm × 75 µm analytical column (Waters). Next, the samples were separated using a 3–40% gradient mobile phase B (0.1% formic acid in acetonitrile) at a flow rate of 300 nL/min, followed by a 20-min rinse with 90% mobile phase B. The data-dependent analysis (DDA) was performed to obtain two analytical replicates for each of three biological sets. This method is used to read a full MS scan in an m/z range of 400–1,600 every 0.5 s, and MS/MS scans (m/z range: 100–1,990) every 0.5 s for the three most intense ions among the full-scan MS. In this context, the protein identification was performed by comparison with the International Protein Index (IPI) bovine database (v. 3.73; 30,403 entries) using the MASCOT search engine v. 2.4 (Matrix Science, Boston, MA, USA), using trypsin as the digestion enzyme, with a parent ion tolerance of 0.2 Da and fragment ion mass tolerance of 0.1 Da. Notably, there were two missed cleavages which were noted and were allowed during trypsin digestion. Additionally, Oxidation (Met) and Methylthio (Cys) were specified as the modification settings. It is noted that the proteins identified with > 95% probability were filtered out. To evaluate the false discovery rate (FDR) of protein identification, data were searched against a combined database of normal and decoy data created by MASCOT. At that time, it is noted that the FDRs of all experiments in this study were < 1%. Hence, the emPAI score of each protein was further used to calculate its relative ratio [19].

2.6 Statistical Analyses

Statistical analyses were performed using SAS v. 9.4 software (SAS Institute, Cary, NC, USA). In this study, the dose responses were analyzed by calculating polynomial orthogonal contrasts (linear, quadratic, cubic) of responses to the addition of L-Ile. Likewise, the differences among treatments were evaluated using the least significant difference (LSD) test. Finally, it is noted that the statistical significance was determined at a level of $P < 0.05$.

Protein upregulation and downregulation were detected using the semi-quantification relative ratio, at cut-off levels of >2 and <0.5, respectively. In this process, the detected proteins were analyzed using a built-in program within the PANTHER online tool (<http://www.pantherdb.org>) for *Bos taurus* pathway analysis. As a consequence, the programs use built-in tools to apply Student's t-test to compare means between the treatments and control at a significance level of $P < 0.05$.

3. Results

In this study, we compared the effects of L-Ile on protein synthesis and the expression levels of β-casein mRNA and proteins.

3.1 AA Time and Dosage Sampling

Intracellular protein (cell protein) in all concentrations peaked at 24 h, and then a continuous decreasing pattern was noted until the timeframe of the 96h (Figure 1a). But in terms of extracellular protein (medium protein) when L-Ile was added, all concentration peaked at 48h (Figure 1). Thus, it

was concluded that the 48 h was the optimal incubation time for the secretion of medium protein by MAC-T cells, indicating that the MAC-T cells were completely differentiated into β -casein-secreting cells. This outcome suggests that 48 h should be adopted as the optimum incubation time for further tests of L-Ile efficacy for the measurement of protein synthesis.

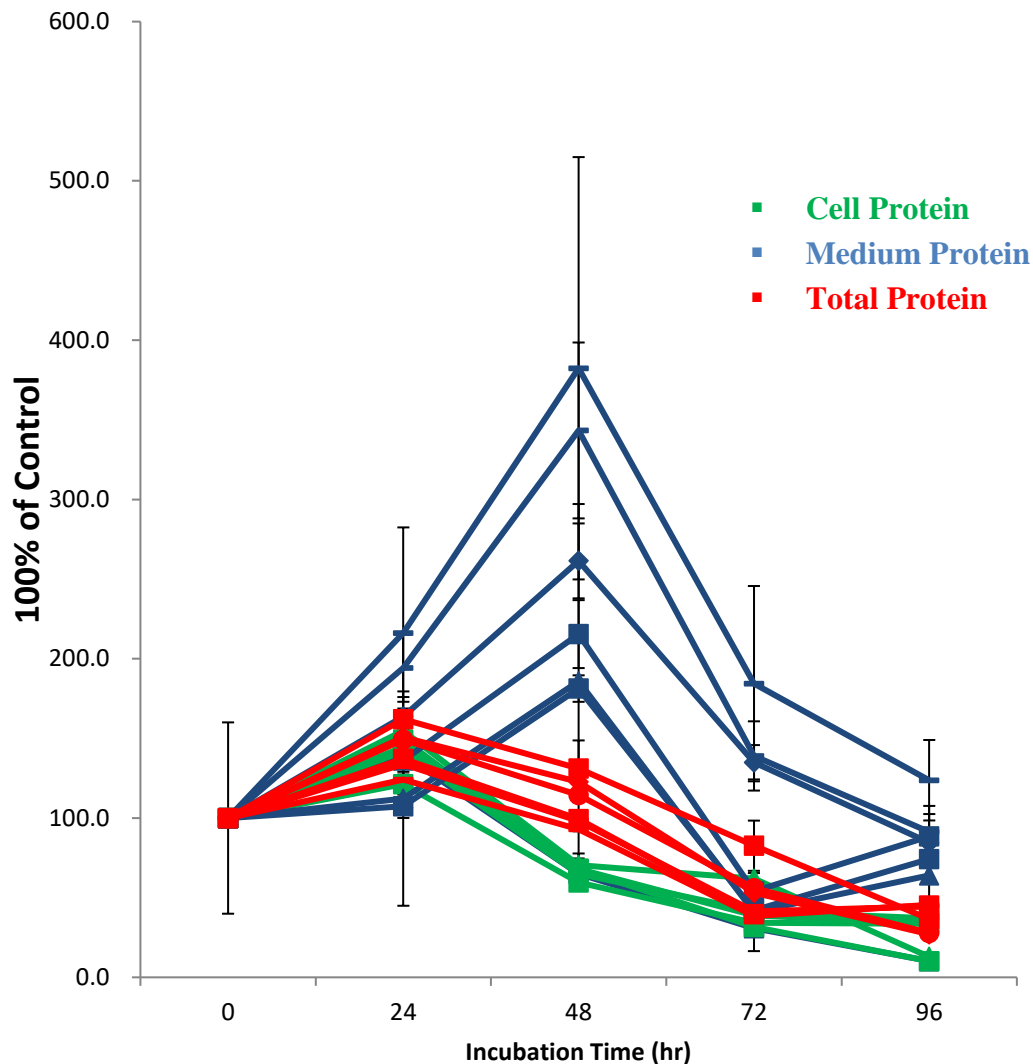


Figure 1. Cell protein, medium protein and total protein in an immortalized bovine mammary epithelial (MAC-T) cell line incubated for 0, 24, 48, 72 and 96 h with different levels of L-Isoleucine (L-Ile) supplementation: Control (+0 mM), +0.3 mM, +0.6 mM, +0.9mM, +1.2 mM, and 1.5 mM. Values are means \pm standard error (SE). For each group, n = 6.

3.2 Protein Content Increased in Response to L-Ile Supplementation

Confirmatory testing noted that extracellular protein (medium protein) showed that 0.6 mM produced the highest medium protein (Figure 2). As we all know, since casein is a protein being secreted outside the cell, the medium protein was chosen as an appropriate basis for selection. It was determined that 0.6 mM dosage of L-Ile is considered as optimal dosage at 48h. Ile, in combination with Leu, may function to stimulate milk protein synthesis [6]. This can occur because supplementation of Ile partitions AAs from muscle protein synthesis, and moves it more towards contributing to the process of milk protein synthesis [7].

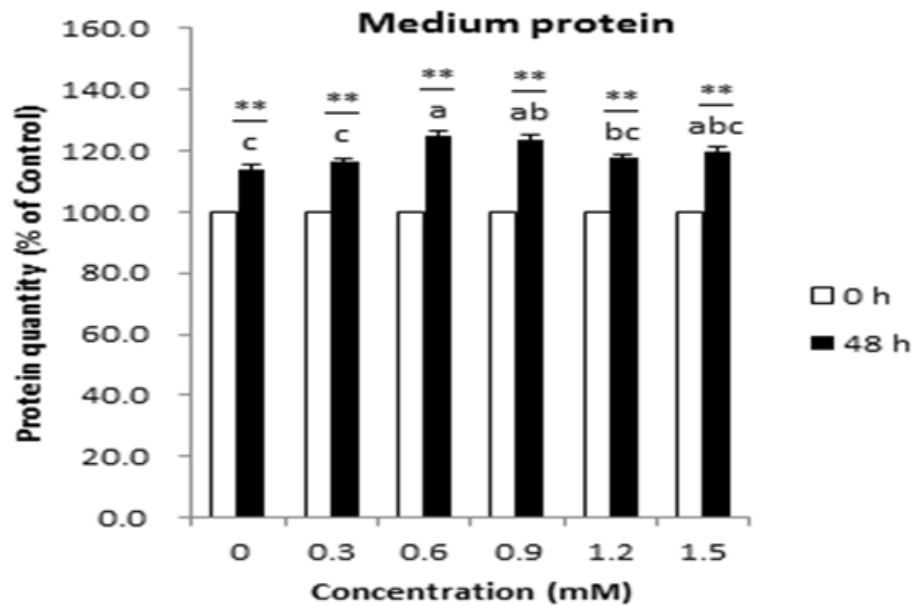


Figure 2. Changes in Extracellular Protein Levels by L-Isoleucine Addition in MAC-T Cells at 0 and 48 hours.

The figure shows the relative % increased of extracellular protein relative to 0H

** T-test between 0h and 48h at each concentration (* P <0.05, ** P <0.01)

3.3 β -casein Expression and Genes Related to Milk Protein Synthesis were Stimulated in Response to L-Ile Supplementation

At 0.6 mM supplementation, L-Ile also showed that it can stimulate β -casein expression (Figure 3). This result indicates that L-Ile was an effective supplement for stimulating β -casein gene expression. It is previously reported that Ile supplementation alone can increase the casein fractional synthesis rate (CFSR) ([8,9]. That being noted, it is concurrently shown that AAs not only function as substrates for synthesis of protein, but also serve as signaling regulatory molecules [3]. Also, the availability of these AAs for mammary epithelial cells is a major limiting factor for protein synthesis, and it is shown as a critical component for the regulation of translation [21,22,23].

Supplementation of L-Ile is also a factor which stimulated mTOR, RPS6 and LDH-B gene expressions. Previous studies showed that Ile supplementation increased mTOR phosphorylation [8,9]. On the contrary, omitting L-Ile from the media reduced mTOR and ribosomal protein S6 (rpS6) phosphorylation in MAC-T cells [9]. In another study, L-Ile supplementation also increased S6K1 and rpS6 phosphorylation [13]. By the same token, it is also reported that the L-Ile linearly increased rpS6 phosphorylation [14]. In this way, the addition of L-Ile also stimulated LDH-B gene expression. Unpublished data from our laboratory demonstrates the role of L-Leu in stimulating LDH-B. But as far as we know, this is the first time the role of L-Ile in stimulating LDH-B has been reported. LDH-B has been known to be involved in the energy metabolism pathway.

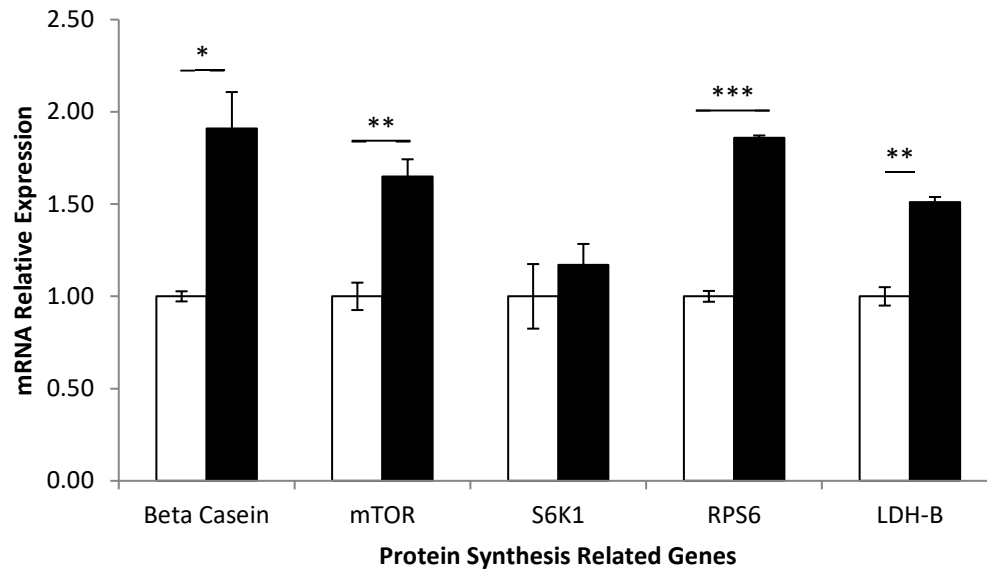


Figure 3. Changes in Gene Expression Levels by 0.6 mM L-Isoleucine Addition in MAC-T Cells at 0 and 48 hours.

*** T-test between 0mM and 0.6mM additional L-Isoleucine at 48h, (* P <0.05, ** P <0.01, *** P <0.001).

3.4 Proteomics Analysis of Milk Protein Synthesis-related Pathways

The total numbers of upregulated and downregulated proteins in the L-Ile treatment group were noted at 63 and 52 (Table 1). Generally speaking, there were several proteins involved in the protein and energy metabolism which exhibited a differential expression following the supplementation with L-Ile. Of note was the insulin receptor-related protein (INSRR) which is an upstream target of mTOR was upregulated, whereas 40S ribosomal protein S2 (RPS2), 60S acidic ribosomal protein P0 (RPLP0), and 60S ribosomal protein L11 (RPL11) were downregulated, by the addition of L-Ile (Table 1). As an important machinery for the process of protein synthesis, RPS2 is a ribosomal protein component of the 40S subunit, while the RPLP0 and RPL11 is a ribosomal protein component of the 60S subunit [24]. These downtrend trends in RPS2, RPLP0, and RPL11 ribosomal protein components are consistent with those that have been reported in past studies. Previous studies have also suggested that the protein export and transformation of AA to tRNA will highlight an increase. It was reported a decreased or unchanged ribosomal component expression despite an increase in milk protein synthesis especially when translation was reduced during lactation. [25]. This findings suggest that decreasing or maintaining the amount of ribosomal components while increasing casein expression and secretion is a mechanism by which the mammary gland can prioritize mRNA translation coding for proteins related to milk synthesis over non-milk-specific proteins, such as ribosomes allowing cells to synthesize milk protein at full capacity [25].

In another study, there was an increase in β -globulin expression, a major milk protein component in mice, but resulted in no change in total milk protein levels in this case [26]. This indicates that the protein synthesis machinery in the lactating mammary tissues was already working in a full capacity as measured by this study. The protein synthesis machinery in the mammary gland decreases adaptably during lactation, which acts as an agent to promote increases for the competitive advantage for casein synthesis [25].

The protein and energy metabolism-related pathways affected by L-Ile supplementation are listed in Table 2. It is shown that the energy metabolism-related pathways such as glycolysis, pentose phosphate pathway and ATP synthesis were stimulated by L-Ile treatment. In addition, insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade pathway, as well

Table 1. Differentially expressed proteins in MAC-T¹ cells supplemented with L-Ile compared with control.

Detected proteins		
Increasing number		63
Decreasing number		52
Selected downregulated and upregulated proteins		
Elongation factor 1-alpha 1	EEF1A1	▼
Eukaryotic translation elongation factor 1 alpha 1-like	LOC784131	▲
Ubiquitin-40S ribosomal protein S27a	RPS27A	▼
Eukaryotic translation elongation factor 1 alpha 1-like	LOC100336381	▼
Elongation factor 2	EEF2	▼
Fatty acid synthase	FASN	▼
Glycogen phosphorylase, liver form	PYGL	▼
Insulin receptor-related protein	INSRR	▲
Eukaryotic translation initiation factor 4A1	EIF4A1	▼
ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha subunit 1	ATP5A1	▼
Ribosomal protein S25-like	LOC785705	▲
Elongation factor 1-gamma	EEF1G	▼
60S acidic ribosomal protein P0	RPLP0	▼
60S ribosomal protein L11	RPL11	▼
40S ribosomal protein S2	RPS2	▼
Zinc finger protein 624	ZNF624	▲
Protein Wnt-2	WNT2	▲

▲: Upregulated (> 2-fold greater protein expression than in control)

▼: Downregulated (< 0.5-fold greater protein expression than in control)

¹MAC-T: Immortalized bovine mammary epithelial (MAC-T) cell line

as the insulin/IGF pathway-protein kinase B signaling cascade pathway, which are upstream target of mTOR were affected by L-Ile supplementation. As we know, milk protein synthesis is a metabolic process demanding high energy requirements, therefore the increase in pathways related to energy and protein metabolism is expected in this case.

Table 2. Protein and energy metabolism-related pathways affected by supplementation of L-Ile¹ compared with control.

Detected pathway*
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade
Insulin/IGF pathway-protein kinase B signaling cascade
p53 pathway
De novo purine biosynthesis
Wnt signaling pathway
Glycolysis
Pentose phosphate pathway
ATP synthesis

* Significantly increased protein and energy metabolism-related pathways ($P < 0.05$) compared with control, as determined by the PANTHER online tool for *Bos taurus* (see Methods for detailed explanation).

¹ L-Ile: L-Ile

4. Discussion

To illustrate the overall effect of L-Ile supplementation in MAC-T cells, a diagram was created to demonstrate the relationships among various components in the synthesis of milk protein (Figures 4). In this context, it is shown that L-Ile supplementation stimulated mTOR, RPS6, and LDH-B gene expression, as well as INSR protein expression. Additionally, the energy synthesis-related pathways such as glycolysis, pentose phosphate pathway, and ATP synthesis were stimulated. In this respect, the protein synthesis-related pathways such as insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade pathway and insulin/IGF pathway-protein kinase B signaling cascade pathway, which are the identified upstream target of mTOR and were also stimulated by L-Ile supplementation. Taken together, the stimulation of genes, proteins and energy/protein metabolic pathways related to protein synthesis elevated β -casein gene expression and eventually increasing protein synthesis.

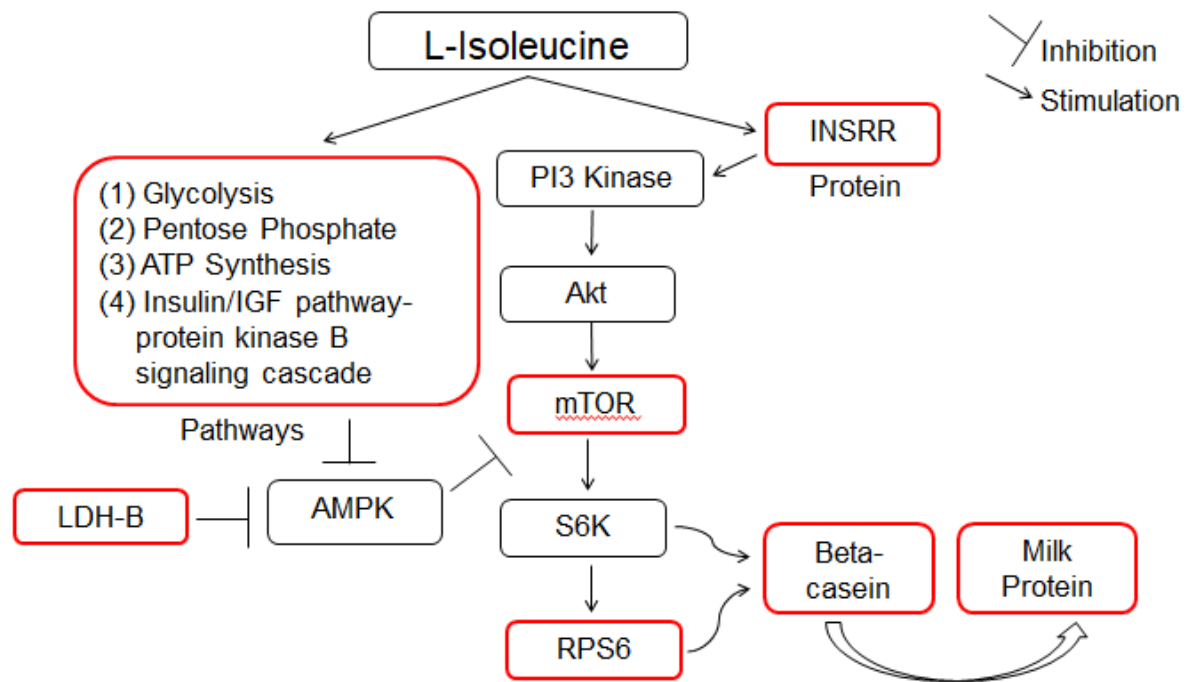


Figure 4. Diagram of the effect of L-Isoleucine supplementation on milk protein synthesis pathway.

Legend: INSRR = Insulin receptor-related protein

LDH-B = lactate dehydrogenase B

IGF = Insulin growth factor

AMPK = AMP-activated protein kinase

PI3 Kinase = Phosphoinositide 3-kinase

Akt = Protein kinase B

mTOR = mammalian target of rapamycin

S6K = S6 Kinase

RPS6 = Ribosomal Protein S6

5. Conclusions

The results of the present study show that 0.6 mM L-Ile supplementation was the optimum dosage in stimulating β -casein mRNA expression by stimulating the expression of genes related to milk protein synthesis, and the increased production of the proteins involved in energy and protein synthesis pathways. Protein and energy metabolism-related pathways were upregulated in the L-Ile supplemented treatment, responsible for and eventually causing the increase in protein concentration in MAC-T cell medium supplemented with L-Ile. In conclusion, L-Ile was effective in increasing protein synthesis in MAC-T cells in vitro by stimulating the genes, proteins and metabolic pathways involved in energy and protein synthesis.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results. Appendix A

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