# Effects of Fatty Acids on Milk Protein and Milk Fat Synthesis-Related Gene Expression in Dairy Cow Mammary Epithelial Cells: Postprint

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## Abstract

This study aimed to explore the combined supplementation patterns of short-chain fatty acids (acetate, -hydroxybutyrate) and long-chain fatty acids (oleic acid, linoleic acid, linolenic acid) for promoting milk protein and milk fat synthesis in bovine mammary epithelial cells (BMECs), thereby providing a theoretical basis for regulating milk composition synthesis. After isolation and purification, BMECs at passage 2 were divided into 5 groups: the control group received no fatty acid supplementation; Groups I and II were supplemented with acetate and -hydroxybutyrate at a concentration ratio of 2.0 (9.60 mmol/L) 1.0 (4.80 mmol/L), and oleic acid, linoleic acid, and linolenic acid at concentration ratios of 2.0 (17.30 mol/L) 13.3 (115.05 mol/L) 1.0 (8.65 mol/L) and 9.6 (75.20 mol/L) 7.4 (58.00 mol/L) 1.0 (7.80 mol/L), respectively; Groups III and IV were supplemented with acetate and -hydroxybutyrate at a concentration ratio of 1.0 (7.20 mmol/L) 1.0 (7.20 mmol/L), and oleic acid, linoleic acid, and linolenic acid at concentration ratios of 2.0 13.3 1.0 and 9.6 7.4 1.0, respectively; the total concentration of short-chain fatty acids (SCFA) and long-chain fatty acids (LCFA) supplemented to each group was 14.541 mmol/L, with 3 replicates per group. After 24 h of culture, the relative growth rate (RGR), triglyceride (TAG) synthesis, and expression levels of genes related to milk protein and milk fat synthesis were measured. The results showed that: 1) RGR and TAG synthesis in BMECs of the treatment groups were significantly higher than those of the control group ($P<0.05$); Group I had the highest RGR and the greatest TAG synthesis. 2) Compared with the control group, Group II significantly increased the expression levels of ribosomal protein S6 kinase 1 (S6K1) and -casein (CSN3) genes ($P<0.05$); Group IV significantly increased the expression level of eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) gene ($P<0.05$); the expression level of signal transducer and activator of transcription 5 (STAT5) gene was significantly decreased in the treatment groups

(P<0.05). 3) Compared with the control group, the expression level of diacylglycerol acyltransferase 2 (DGAT2) gene was significantly increased (P<0.05), while the expression level of fatty acid synthase (FASN) gene was significantly decreased (P<0.05) in the treatment groups. In conclusion, supplementation of the culture medium with 7.20 mmol/L acetate, 7.20 mmol/L -hydroxybutyrate, 75.20 mol/L oleic acid, 58.00 mol/L linoleic acid, and 7.80 mol/L linolenic acid had a favorable promoting effect on the expression levels of genes related to milk protein and milk fat synthesis in BMECs.

## Full Text

# Effects of Fatty Acids on Expression Levels of Genes Involved in Milk Protein and Milk Fat Synthesis in Bovine Mammary Epithelial Cells

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### Abstract

This study aimed to explore optimal combined supplementation patterns of short-chain fatty acids (acetic acid, -hydroxybutyric acid) and long-chain fatty acids (oleic acid, linoleic acid, linolenic acid) to promote milk protein and milk fat synthesis in bovine mammary epithelial cells (BMECs), thereby providing a theoretical basis for regulating milk composition synthesis. BMECs were isolated and purified, and second-passage cells were divided into five groups with three replicates each. The control group received no fatty acid supplementation. In groups I and II, the concentration ratio of acetic acid to -hydroxybutyric acid was 2.0 (9.60 mmol/L) : 1.0 (4.80 mmol/L), while the ratios of oleic acid, linoleic acid, and linolenic acid were 2.0 (17.30 μmol/L) : 13.3 (115.05 μmol/L) : 1.0 (8.65 μmol/L) and 9.6 (75.20 μmol/L) : 7.4 (58.00 μmol/L) : 1.0 (7.80 μmol/L), respectively. In groups III and IV, the acetic acid to -hydroxybutyric acid ratio was 1.0 (7.20 mmol/L) : 1.0 (7.20 mmol/L), with oleic acid, linoleic acid, and linolenic acid ratios of 2.0 : 13.3 : 1.0 and 9.6 : 7.4 : 1.0, respectively. The total concentrations of short-chain fatty acids (SCFAs) and long-chain fatty acids (LCFAs) were 14.541 mmol/L and 141 μmol/L, respectively. After 24 hours of culture, cell relative growth rate (RGR), triglyceride (TAG) synthesis, and expression levels of genes related to milk protein and milk fat synthesis were measured.

The results showed: (1) RGR and TAG synthesis in all treatment groups were significantly higher than in the control group (P < 0.05), with group I showing the highest RGR and maximum TAG synthesis. (2) Compared with the control, group II significantly increased expression of ribosomal protein S6 kinase 1 (S6K1) and -casein (CSN3) genes (P < 0.05); group IV significantly increased expression of eukaryotic translation initiation factor 4E binding protein

1 (4EBP1) gene ($P < 0.05$); and all treatment groups showed significantly decreased expression of signal transducer and activator of transcription 5 (STAT5) gene ($P < 0.05$). (3) Compared with the control, diacylglycerol acyltransferase 2 (DGAT2) gene expression was significantly increased in all treatment groups ($P < 0.05$), while fatty acid synthase (FASN) gene expression was significantly decreased ($P < 0.05$).

In conclusion, supplementation with 7.20 mmol/L acetic acid, 7.20 mmol/L -hydroxybutyric acid, 75.20 µmol/L oleic acid, 58.00 µmol/L linoleic acid, and 7.80 µmol/L linolenic acid effectively promoted expression of genes involved in milk protein and milk fat synthesis in BMECs.

**Keywords:** bovine mammary epithelial cells; short-chain fatty acids; long-chain fatty acids; milk protein; milk fat

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Milk protein and milk fat are critical indicators of milk quality. Acetic acid and -hydroxybutyric acid, derived from rumen fermentation, serve as primary precursors for de novo fatty acid synthesis in bovine mammary epithelial cells (BMECs), with approximately 50% of C16 fatty acids and long-chain fatty acids (LCFAs) originating from dietary sources and body fat mobilization [1]. Kong [2] reported that adding acetic acid and butyric acid to BMECs increased expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) genes, enhanced triglyceride (TAG) synthesis, and upregulated expression of mammalian target of rapamycin (mTOR) and diacylglycerol acyltransferase 2 (DGAT2) genes. Liang et al. [3] demonstrated that low concentrations of oleic acid promoted expression of ACC and FASN genes, thereby stimulating milk fat synthesis. Li [4] found that when oleic acid, linoleic acid, and linolenic acid were supplied at a ratio of 2 (22.20 µmol/L) : 13.3 (147.70 µmol/L) : 1 (11.10 µmol/L), expression of S1-casein (CSN1S1), -casein (CSN3), and mTOR genes was upregulated in BMECs. These findings indicate that fatty acids play crucial regulatory roles in lactation and significantly influence milk protein and milk fat synthesis.

Mach et al. [5] reported that dietary supplementation with rapeseed, soybean, and flaxseed increased milk yield but decreased milk fat and protein content, suggesting that the proportion of unsaturated fatty acids in milk fat precursors (MFP) may regulate milk fat synthesis. While previous studies have advanced understanding of milk secretion mechanisms and nutritional regulation of milk composition, the molecular biological mechanisms and signal transduction pathways underlying nutrient regulation of milk protein and fat synthesis remain unclear. Most research has focused on single fatty acid supplementation, with limited studies investigating combined effects of short-chain fatty acids (SCFAs) and LCFAs on milk component synthesis in BMECs. Therefore, this study examined the effects of mixed supplementation with different ratios of acetic acid, -hydroxybutyric acid, oleic acid, linoleic acid, and linolenic acid on expression of genes related to milk protein and fat synthesis in BMECs, aiming to identify op-

timal fatty acid combination patterns for promoting milk component synthesis and providing theoretical basis for nutritional regulation.

## 1. Materials and Methods

### 1.1 Isolation and Purification of BMECs

Mammary tissue samples were collected from healthy Chinese Holstein cows in peak lactation. Approximately 55 g of mammary tissue rich in alveoli was excised and placed in phosphate-buffered saline (PBS) containing antibiotics. Primary BMECs were obtained through type II collagenase digestion and cultured in a humidified incubator at 37 °C with 5% CO . After approximately one week, when cells reached 80% confluence, BMECs were purified based on differential trypsin digestion times for fibroblasts and epithelial cells, then passaged. Second-passage BMECs were used for experiments.

### 1.2 Culture Medium

The experimental culture medium consisted of DMEM/F12 supplemented with hydrocortisone, glutamine, insulin-transferrin-selenium, and prolactin. All reagents were purchased from GIBCO.

### 1.3 Experimental Design

This study employed a single-factor completely randomized design. Fatty acid concentration ratios were based on previous studies by Li [4] and Ta et al. [6], which demonstrated that an acetic acid to -hydroxybutyric acid ratio of 2.0:1.0 combined with an oleic acid to linoleic acid to linolenic acid ratio of 2.0:13.3:1.0 effectively promoted expression of milk fat synthesis-related genes, while a 1.0:1.0 ratio of acetic acid to -hydroxybutyric acid combined with 9.6:7.4:1.0 ratio of oleic acid to linoleic acid to linolenic acid favored milk protein synthesis-related gene expression.

Accordingly, five treatment groups were designed with identical total concentrations of SCFAs (acetic acid and -hydroxybutyric acid; total 14.4 mmol/L) and LCFAs (oleic acid, linoleic acid, and linolenic acid; total 141 μmol/L) but different ratios among individual fatty acids. The experimental design is detailed in Table 1 .

### 1.4 Analytical Methods

**1.4.1 Cell Viability Assessment** Cell viability was measured using the thiazolyl blue tetrazolium bromide (MTT) colorimetric assay. BMECs were seeded in 96-well plates at a density of $1 \times 10$ cells per well and cultured until reaching approximately 80% confluence. Cells were then treated with induction media containing different fatty acid ratios for 24 hours, with three replicates per group. Four hours before the end of culture, 20 μL of MTT solution (5 mg/mL) was added to each well. After incubation, the supernatant was removed, 100

μL of dimethyl sulfoxide (DMSO) was added, and plates were shaken for 10 minutes. Absorbance at 490 nm (OD ) was measured using an automated microplate reader. Relative growth rate (RGR) was calculated as: RGR = OD of treatment group / OD of control group.

**1.4.2 TAG Synthesis Measurement** Second-passage BMECs were seeded in 75 $cm^2$ culture flasks at approximately $8 \times 10$ cells per flask in 10 mL of cell suspension. After reaching 80% confluence, cells were treated with induction media containing different fatty acid ratios and cultured for an additional 24 hours (three replicates per group). Cells were then digested with trypsin/EDTA, centrifuged for 8 minutes, washed once with PBS, and intracellular TAG content was measured using a TAG assay kit.

**1.4.3 Gene Expression Analysis** Second-passage BMECs were cultured until reaching 70% confluence, then treated with induction media containing different fatty acid ratios for 24 hours (three replicates per group). After treatment, cells were collected for RNA extraction using RNAiso Plus reagent, followed by reverse transcription using PrimeScript™ RT Reagent Kit. Expression levels of genes related to milk protein synthesis (CSN1S1, CSN3), signaling pathway components [mTOR, STAT5, 4EBP1, S6K1, protein kinase B (AKT)], milk fat synthesis (ACC, FASN, DGAT2), and transcriptional regulators [peroxisome proliferator-activated receptor (PPAR ), sterol regulatory element-binding protein 1 (SREBP1)] were quantified by RT-qPCR using TaKaRa reagents. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference gene. Primer sequences and parameters are listed in Table 2 . Gene expression levels were calculated using the 2^(-ΔΔCt) method [6].

### 1.5 Statistical Analysis

Experimental data were processed and organized using Excel 2007. RT-qPCR results were analyzed using the 2^(-ΔΔCt) method for relative quantification. Data were subjected to ANOVA using SAS 9.0 software. Differences were considered significant at $P < 0.05$.

## 2. Results

### 2.1 Effects of Different Fatty Acid Ratios on RGR and TAG Synthesis in BMECs

As shown in Table 3 , RGR and TAG synthesis in all treatment groups were significantly higher than in the control group ($P < 0.05$). Group I exhibited the highest RGR and maximum TAG synthesis, significantly exceeding other treatment groups ($P < 0.05$). Groups II and IV showed significantly higher values than group III ($P < 0.05$), with no significant difference between groups II and IV ($P > 0.05$).

### 2.2 Effects of Different Fatty Acid Ratios on Expression of Milk Protein Synthesis-Related Genes in BMECs

Table 4 shows that compared with the control, group I significantly decreased expression of CSN1S1, CSN3, and STAT5 genes ($P < 0.05$) while significantly increasing mTOR and 4EBP1 gene expression ($P < 0.05$). Groups II, III, and IV significantly upregulated CSN3 and S6K1 expression ($P < 0.05$) but downregulated STAT5 expression ($P < 0.05$). Groups III and IV showed significantly increased AKT and 4EBP1 expression ($P < 0.05$), while group II exhibited significantly decreased mTOR and 4EBP1 expression ($P < 0.05$).

### 2.3 Effects of Different Fatty Acid Ratios on Expression of Milk Fat Synthesis-Related Genes in BMECs

As presented in Table 5 , groups II, III, and IV showed significantly higher ACC expression compared with the control ($P < 0.05$), while all treatment groups exhibited significantly increased DGAT2 expression ($P < 0.05$). ACC expression in groups III and IV was significantly higher than in groups I and II ($P < 0.05$), with group II significantly exceeding group I ($P < 0.05$). DGAT2 expression in groups II and IV was significantly higher than in groups I and III ($P < 0.05$), with group III significantly higher than group I ($P < 0.05$). All treatment groups showed significantly decreased expression of FASN and SREBP1 genes ($P < 0.05$). PPAR  expression was significantly increased in group I ($P < 0.05$) but decreased in other treatment groups ($P < 0.05$).

## 3. Discussion

### 3.1 Effects of Different Fatty Acid Ratios on BMEC Proliferation

Fatty acids represent essential nutrients in mammary tissue that influence BMEC growth and proliferation. Qi et al. [7] reported that low concentrations of acetic acid promoted BMEC proliferation, whereas high concentrations were inhibitory. Sun [8] demonstrated that oleic acid at 50–400 μmol/L and linoleic acid at 25–100 μmol/L stimulated BMEC proliferation, but concentrations of 800 μmol/L and 200 μmol/L, respectively, became inhibitory. Yonezawa et al. [9] found that 100 μmol/L oleic and linoleic acids promoted BMEC proliferation, while other studies indicated that oleic acid concentrations above 0.20 mmol/L suppressed cell proliferation [10-11].

AKT contributes to cell survival and proliferation [12]. In this study, AKT expression patterns correlated with RGR changes, though the underlying mechanisms require further investigation.

### 3.2 Effects of Different Fatty Acid Ratios on Expression of Milk Protein Synthesis-Related Genes

The Janus kinase 2 (JAK2)/STAT5 signaling pathway is primarily regulated through phosphorylation of receptor-bound STAT5 by JAK, enabling activated

STAT5 dimers to translocate to the nucleus and modulate target gene transcription [13]. Bionaz and Loor [14] found no significant changes in STAT5 and JAK expression during lactation, suggesting limited regulatory impact on milk protein synthesis. In this study, all treatment groups showed significantly lower STAT5 expression than the control, while groups III and IV exhibited significantly higher 4EBP1 and S6K1 expression. The inconsistent pattern between STAT5 expression and CSN1S1/CSN3 expression suggests that milk protein gene expression results from integrated regulation by both mTOR and JAK2/STAT5 signaling pathways.

CSN1S1 and CSN3 are two major casein genes strongly correlated with milk protein synthesis in BMECs [15]. Yonezawa et al. [16] reported that 400 μmol/L oleic and linoleic acids promoted CSN1S1 expression, while Pauloin et al. [17] found that unsaturated fatty acids decreased CSN3 expression in mouse mammary epithelial cells. In this study, CSN1S1 expression in group IV was higher than the control (though not significantly), while group II showed the highest CSN3 expression, indicating that an oleic acid:linoleic acid:linolenic acid ratio of 9.6:7.4:1.0 significantly promoted CSN1S1 and CSN3 expression.

mTOR is an atypical serine/threonine protein kinase [18-19] that regulates milk protein synthesis, cellular energy status, and amino acid utilization in mammary tissue, in addition to endocrine signaling [20]. Upstream, mTOR is positively regulated by AKT phosphorylation, while downstream it promotes phosphorylation of S6K1 and 4EBP1 to regulate translation initiation. These two downstream pathways operate in parallel, controlling mRNA translation of specific subunits [21]. Additionally, mTOR signaling inhibits expression of SREBP1 target genes ACC and FASN, indicating mTOR involvement in de novo fatty acid synthesis [22]. This study found that groups I and III showed significantly higher mTOR, AKT, and 4EBP1 expression than the control, suggesting that an oleic acid:linoleic acid:linolenic acid ratio of 2.0:13.3:1.0 upregulated mTOR, AKT, 4EBP1, and S6K1 expression, while an acetic acid: -hydroxybutyric acid ratio of 2.0:1.0 inhibited S6K1 expression. The mechanisms underlying mixed fatty acid regulation in BMECs warrant further investigation.

### 3.3 Effects of Different Fatty Acid Ratios on Expression of Milk Fat Synthesis-Related Genes and TAG Synthesis

Both AKT and mTOR signaling pathways regulate milk protein synthesis and promote SREBP signaling, which subsequently modulates expression of target genes ACC and FASN to influence milk fat synthesis. ACC and FASN are two crucial genes in de novo milk fat synthesis [23]. Ma et al. [24] demonstrated that 100 nmol/L SREBP1 siRNA decreased ACC and FASN mRNA levels in BMECs. In this study, treatment groups showed significantly higher AKT and ACC expression but lower FASN and SREBP1 expression compared with the control, suggesting that mixed fatty acids induced negative feedback regulation of SREBP1 by the AKT pathway, with SREBP1 positively regulating FASN while negatively regulating ACC.

Numerous studies have identified SREBP1 and PPAR as important nuclear receptors and transcriptional regulators of milk fat synthesis-related genes, with PPAR potentially participating in SREBP1 regulation. PPAR occupies a central position in adipogenic regulatory pathways, modulating gene expression by influencing fatty acid uptake, transport, de novo synthesis, and esterification [25]. This study found that PPAR expression was higher in group I but lower in other treatment groups compared with the control, suggesting PPAR may be involved in SREBP1 regulation while being modulated by AKT and mTOR.

DGAT2 is an integral microsomal enzyme in the endoplasmic reticulum that plays a vital role in catalyzing TAG synthesis, serving as the sole rate-limiting enzyme in this process [26]. This study showed that groups II and IV had higher DGAT2 expression than the control, accompanied by significantly increased TAG synthesis, demonstrating DGAT2' s regulatory role in TAG synthesis. Milk fat consists primarily of TAG derived from chylomicrons in BMECs; thus, TAG synthesis directly reflects milk fat synthesis capacity. Fatty acids regulate TAG synthesis through the aforementioned signaling pathways. Cui [27] reported that TAG synthesis in BMECs positively correlated with various C18 fatty acid concentrations under non-inhibitory conditions (0–10 μmol/L). Ta et al. [6] found that appropriate acetic acid: -hydroxybutyric acid ratios (2.0:1.0 and 4:1) promoted TAG synthesis and expression of milk fat synthesis-related genes. Shng et al. [28] reported that an oleic acid:linoleic acid:linolenic acid ratio of 2.0:13.3:1.0 enhanced TAG synthesis in BMECs. The current results showing significantly higher TAG synthesis in all treatment groups confirm that supplementation with appropriate fatty acid ratios promotes intracellular TAG synthesis.

In summary, supplementation with acetic acid, -hydroxybutyric acid, oleic acid, linoleic acid, and linolenic acid at concentrations of 7.20 mmol/L, 7.20 mmol/L, 75.20 μmol/L, 58.00 μmol/L, and 7.80 μmol/L, respectively, effectively increased expression of CSN1S1, CSN3, AKT, 4EBP1, S6K1, and DGAT2 genes, thereby promoting milk protein and milk fat synthesis in BMECs.

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*Note: Figure translations are in progress. See original paper for figures.*