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Article

The Effects of *trans*-10, *cis*-12 Conjugated Linoleic Acid on the Production Performance of Dairy Cows and the Expression and Transcription Regulation of Lipid Metabolism-Related Genes in Bovine Mammary Epithelial Cells

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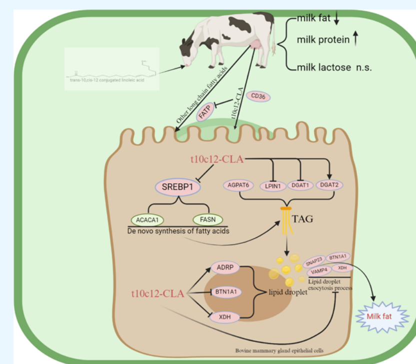
ABSTRACT: Dietary fatty acids (FAs) determine the quality of dairy products. The *trans*-10, *cis*-12 conjugated linoleic acid (*t*10*c*12-CLA) is commonly considered an FA factor leading to milk fat depression syndrome (MFDs) in dairy cow. However, its effect on dairy cow performance and involvement in milk fat metabolism have been insufficiently explored. This study administered 136.17 g/day of rumen-protected CLA (RP-CLA) to dairy cows and found a diminution in milk fat percentage and a trend of increasing milk protein percentage on day 21 postpartum. Lactose content, milk yield, and net energy for lactation were unaffected. In the cell experiments, Oil Red O staining showed a notable increase in lipid droplets. Gene and protein expression analysis showed that 300 μM *t*10*c*12-CLA upregulated the expression of CD36, DGAT2, and ADRP, while downregulating the expression of ACACA, FASN, SREBP1, FABP3, FATP3, ACSL4, LPIN1, DGAT1, BTN1A1, XDH, SNAP23, and VAMP4. This provides a possible mechanistic pathway for the contradictory phenomenon of *t*10*c*12-CLA reducing milk fat while increasing lipid droplets. Overall, *t*10*c*12-CLA, as a long-chain fatty acid, can promote lipid droplet synthesis but may reduce milk fat by inhibiting lipid droplet fusion and secretion, FAs de novo synthesis, and triglyceride biosynthesis.



1. INTRODUCTION

In dairy production, the regulation of dietary fatty acids (FAs) is crucial in deciding the proportion and constituent of milk fat.[1−3] Conjugated linoleic acid (CLA) is an unsaturated long-chain fatty acid that exists in multiple geometric isomers due to differing positions of conjugated double bonds Among these isomers, *t*10*c*12-CLA and *c*9*t*11-CLA are the most biologically active,[4] and their mechanisms of action have been thoroughly investigated. Baumgard et al. administered 3.5, 7.0, and 14.0 g of *t*10*c*12-CLA via abomasal infusion to lactating cows, leading to a reduction in milk fat content by 25, 33, and 50%, respectively. They found that c9t11-CLA did not have the equivalent results.[5,6] Peterson et al. treated bovine mammary epithelial cells (BMECs) with 75 μM *t*10*c*12-CLA, resulting in a remarkable diminution in the expression of acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase (ACC, FAS, and SCD) mRNAs that decreased significantly. However, 75 μmol/L c9t11-CLA did not result in notable changes in the expression of the aforementioned genes.[1] Harvatine and Bauman found that administering *t*10*c*12-CLA (10 g/d) via the jugular vein in dairy cows causes a markable diminish in the expression of SREBF1, Insig1, and THRSP genes, as well as mature SREBP proteins in mammary tissue.[7] Kadegowda et al. investigated BMECs and found that both *t*10 C 18:1 and *t*10*c*12-CLA suppressed the expression of FASN, SCD, and Sterol regulatory element-binding protein-1 (SREBP1) genes. They found that *t*10*c*12-CLA concurrently suppressed the expression of acetyl-CoA synthetase short-chain family member 2, fatty acid-binding protein 3, insulin-induced gene 1, and sterol regulatory element-binding protein-2 (ACSS2, FABP3, INSIG1, and SREBP2), which indicated that *t*10*c*12-CLA can reduce the activity of milk fat biosynthesis genes.[8] Experiments conducted on mice showed that *t*10*c*12-CLA can inhibit the pathways of genes related to de novo FAs biosynthesis, desaturation, and triglyceride (TG) synthesis, resulting in a notable diminish in milk fat proportion.[9] In the CLA mixture, *t*10*c*12-CLA exerts a diminishing impact on milk fat and affects multiple pathways in lipid metabolism. The process of milk fat biosynthesis and the genes pivotal to it include de novo synthesis (SREBP1, ACACA1, and FASN),[10,11] uptake, and transport of long-chain fatty acids (LCFAs) (FABP3, FATP3, ACSL1, ACSL4, and CD36) and glycerol.[11] The biosynthesis of TGs (AGPAT6,

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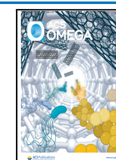

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Table 1. TMR (Unit in g)

molasses	fat powder	DDGS	pelleted corn	calcium fatty acids	premix	cottonseed	soybean meal	domestic Alfalfa	corn bran powder	beet pellets
218	150	700	3600	300	1100	2000	2386	967	600	200
silage	imported Alfalfa	wheat bran	extruded soybeans	corn	rumen probiotics	domestic oat grass	soybean hulls	Alfalfa silage	water	total
25,000	1000	200	543	6366	400	1000	200	2700	5600	55,230

LPIN1, DGAT1, and DGAT2)[11,12] and the synthesis, fusion, and efflux of lipid droplets (BTN1A1, ADRP, XDH, SNAP23, and VAMP4).[13,14] Therefore, understanding the impact of adding *t*10*c*12-CLA on milk fat metabolism in BMECs at the cellular level can reveal its regulatory mechanism at the molecular level. Additionally, the impacts of CLA on milk composition and milk production in cows are inconsistent except for reducing milk fat,[6,15] and research on its impact on net milk production in cows is limited. In this study, the impacts of *t*10*c*12-CLA on milk composition, milk production, and net milk production were first determined in perinatal cows, and the reasons for differences from the results of other published studies were investigated. Second, although studies on how *t*10*c*12-CLA suppresses milk fat have been conducted, they have not comprehensively assessed the specific network mechanisms involved. Therefore, we utilized BMECs to study the different impacts of *t*10*c*12 CLA on the milk fat biosynthesis pathway of BMECs and established the potential links between its different effects. We hope to explain the specific mechanism by which the *t*10*c*12 CLA affects the milk fat biosynthesis pathway.

2. MATERIALS AND METHODS

2.1. In Vivo Experiment. *2.1.1. Preparation of Rumen-Protected CLA (RP-CLA).* In another study,[15] 10 g of *t*10*c*12-CLA was found to exhibit the maximum inhibition rate of milk fat biosynthesis in dairy cows. To ensure efficacy and prevent deficiencies, 15 g of *t*10*c*12-CLA was used in this experiment. Since *t*10*c*12-CLA is prone to saturation by rumen microbiota, a rumen-protected coating was applied to the raw material. The proportions of each component in the conjugated linoleic acid mixture are listed in Supporting Table 1 (Qingdao Aohai Biotechnology Co., Ltd., Shandong, China), where the proportion of *t*10*c*12-CLA was above 36.0%. The coating of the raw materials was performed by Hangzhou Kangdequan Feed Co., Ltd. The coating efficiency and rumen protection rate were determined to be 40 and 85%, respectively. To ensure that 15 g of *t*10*c*12-CLA was administered per cow, 136.17 g of RP-CLA was administered orally.

2.1.2. Ethics Statement. The experiment was conducted at Yanqing Shounong Livestock Breeding Farm in Beijing. The Institutional Animal Care and Use Committee of China Agricultural University reviewed and approved the experimental protocol with approval number AW02113202−2−1.

2.1.3. Animal Husbandry and Management. Primiparous Holstein cows (n = 12) at 7 and 21 days postpartum were allocated into two groups: the control group (n = 6; Body condition score (BCS): 3.25 ± 0.16; mean ± SD) and the CLA group (n = 6; (BCS): 3.29 ± 0.19; mean ± SD), ensuring an even distribution between the groups. Cows in the CLA group were orally administered 136.17 g of RP-CLA daily from days 1 to 7 of the experiment, whereas cows in the control group were provided with a standard diet without CLA. Changes in various parameters were monitored continuously from days 8 to 14 of the experiment. Throughout the experimental period, all cows were provided with a total mixed ration (TMR) twice daily at 7 a.m. and 7 p.m. (see Table 1 for details). Milking was conducted 4 times daily at 6 a.m., noon, 6 p.m., and midnight, and ad libitum access to water was provided.

2.1.4. Data Collection. The data on feeding periods and milk production, including milk yield and milk composition, for all cows within 7 days after cessation of supplementation were collected from the Afifarm Management System and stored until further analysis.

Milk fat accounts for the largest proportion of energy value in milk, as reported by Tyrrell and Reid[16] (total energy output (MJ/kg) = [0.384 × fat percentage + 0.223 × protein percentage + 0.199 × lactose percentage −0.108] × milk yield (kg)).

2.2. In Vitro Experiment. *2.2.1. Chemical Reagent.* *t*10*c*12-CLA (purity ≥98.0%) was sourced from CAYMAN CHEMICAL (Batch: 0565670−30).

2.2.2. Preparation of Different Concentrations of Fatty Acids. A stock solution of 89 mM *t*10*c*12-CLA (CAYMAN CHEMICAL, Batch: 0565670−30) was prepared by dissolving it in anhydrous ethanol. Various concentrations of working solutions (75, 150, 200, 300, and 400 μM) of CLA were prepared by adding the appropriate volume of the stock solution into the medium containing 0.2% (w/v) FFA-free bovine serum albumin.[17] The control group was prepared by adding an equivalent capacity of anhydrous ethanol.

2.2.3. Cell Culture and Sample Treatment. Two types of culture media are required for culturing cells, including a growth medium and a serum-free basic medium. The growth medium consists of DMEM-F12 basic medium (Batch number: 6,124,053; Thermo Fisher Scientific Co., Ltd.) and 10% fetal bovine serum (FBS) (Batch number: FB15016 Clark Australia), and 1% 100 × penicillin-streptomycin mixture (Batch number: P1400, Beijing Solebao Technology Co., Ltd., Beijing, China). The serum-free medium component contains the DMEM-F12 basic medium. Resuscitated bovine mammary alveolar T cells (MAC-T) were inoculated in culture flasks, added to the growth medium, and cultured at 37 °C and 5% CO_2, under saturated humidity. The Culture medium was replaced every 24 h. When the cells reached 80% confluence, relevant experimental treatments were conducted. The culture solution was replaced with serum-free DMEM/F12 12 h before treating with *t*10*c*12-CLA. Subsequently, to the experimental group, a serum-free culture medium containing *t*10*c*12-CLA was added, whereas in the control group, corresponding volumes of anhydrous ethanol were added. Both groups were treated for 24 h

2.2.4. RNA Extraction and Fluorescence Quantitative PCR Detection. Cellular RNA was isolated using the SteadyPure Rapid RNA Extraction Kit (Accurate Biotechnology (Hunan) Co., Ltd.). The purity and concentration of RNA were determined using a NANODROP ONE spectrophotometer (Hangzhou Bioer Technology Co., Ltd., Hangzhou, China) by

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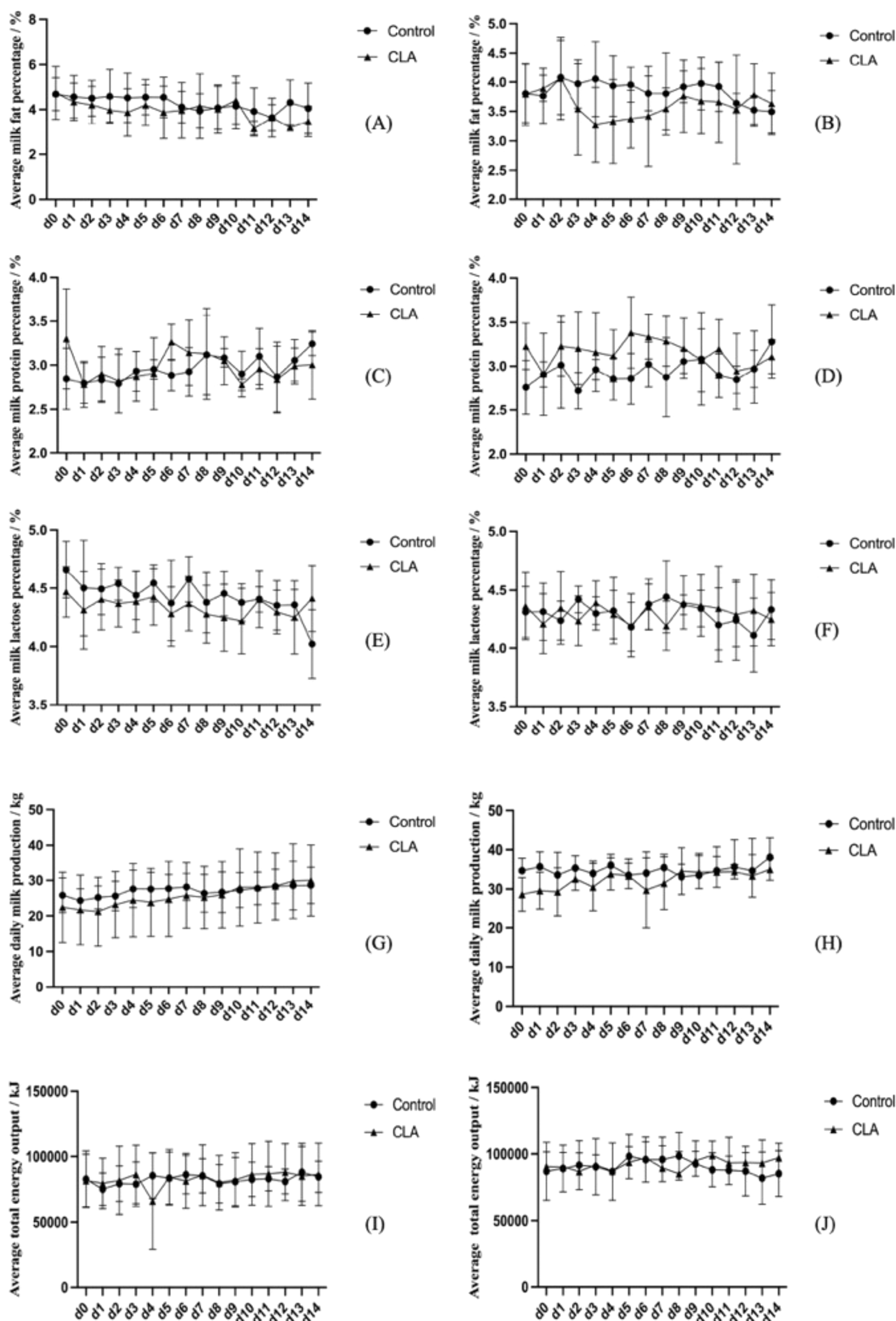

Figure 1. Effects of CLA on the milk composition, milk yield, and milk net energy of cows 7 and 21 days postpartum. (A) CLA effect on milk fat of cows 7 days postpartum; (B) CLA effect on milk fat of cows 21 days postpartum; (C) CLA effect on milk protein of cows 7 days postpartum; (D) CLA effect on milk protein of cows 21 days postpartum; (E) CLA effect on milk lactose of cows 7 days postpartum; (F) CLA effect on milk lactose of cows 21 days postpartum; (G) CLA effect on milk yield of cows 7 days postpartum; (H) CLA effect on milk yield of cows 21 days postpartum; (I) CLA effect on total energy output of cows 7 days postpartum; (J) CLA effect on total energy output of cows 21 days postpartum.

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measuring the OD ratio at 260/280 nm. The A260/A280 and A260/230 ratios were found to be 2.01:2.04 and 1.81:1.95, respectively, indicating that all total RNA samples were pure and free from protein and organic contaminants. Subsequently, 1 μg of cDNA was gained using the reverse transcription kit (Accurate Biotechnology (Hunan) Co., Ltd., Hunan, China), following the operating procedure of the reagent kit. If the next step is not taken immediately, the transcribed cDNA should be stored at −20 °C. For quantitative PCR analysis, the SYBR Green Pro TaqHS Premix qPCR Kit III (with low Rox; Accurate Biotechnology (Hunan) Co., Ltd., Hunan, China) was used in a 7500 Real-Time PCR System (Applied Biosystems by Thermo Fisher Scientific, Weijieji Trading Co., Ltd., Shanghai, China). The relative expression of the mRNA of the target genes was assessed using specific primer sequences listed in Supporting Table 2. Each sample was analyzed with three biological duplicates and three technical duplicates. The mRNA expression was figured by the $2^{-\Delta\Delta CT}$ method.[18,19]

2.2.5. Protein Extraction and Western Blotting. Total protein was extracted from cultured MAC-T cells according to the operating procedure using the Protein Extraction Kit (Batch: P0013B, Beyotime Biotechnology Institute, China). The concentration of protein was assessed by the BCA Protein Quantification Kit (Batch: P1511, Beijing Pulilai Gene Technology Co., Ltd., Beijing, China). After mixing with the sample buffer and boiling for 10 min, it was stored at −20 °C until further use. Cellular proteins were used for SDS-PAGE, and then the proteins were transferred onto 0.2 μm PVDF membranes (Batch: 0000155412, Sigma-Aldrich). After the PVDF membranes were blocked with a 5% skim milk solution (Batch: 508 l051, Beijing Solabio Technology Co., Ltd., Beijing, China) for 2 h at room temperature, the membranes were incubated with primary antibodies at appropriate concentrations for 16 h at 4 °C. All antibodies were purchased from Proteintech, Wuhan Sanying Biotechnology Co., Ltd. unless otherwise stated. The antibodies used were Caspase3 (1:1000, Catalog Number: 19,677−1-AP), Bax (1:10,000, 50,599−2-Ig), Bcl2 (1:1000, 26,593−1-AP), ADRP (1:10,000, 15,294−1-AP), BTN1A1 (1:1000, 26,687−1-AP), XDH (1:1000, 55,156−1-AP), SNAP23 (1:1000, 10,825−1-AP), VAMP4 (1:1000, 10,738−1-AP), and β-actin (1:50,000, AC026, ABclonal, Wuhan Aobote Biotechnology Co., Ltd.). After using Tris-buffered saline and Tween-20 (TBST) solution to wash the membranes thrice (Batch: GC2306002, Wuhan Saiweier Biotechnology Co., Ltd., Wuhan, China) (10 min per wash), and the membranes were incubated with the corresponding secondary antibodies (1:10,000, SA00001−2, Proteintech, Wuhan Sanying Biotechnology Co., Ltd.) for 1 h at room temperature on a shaking table. The β-actin was used as the reference, and the relative expression of the protein was normalized.

2.2.6. Cell Viability Assay. The MAC-T cells were seeded in a 96-well plate at a density of 10,000 cells per well and cultured until the cells reached approximately 80% confluence (approximately 24 h). Next, the cells were treated with serum-free DMEM for 12 h. Subsequently, the cells were treated with different concentrations of *t*10*c*12-CLA (0, 75, 150, 200, 300, and 400 μmol/L) for 4, 12, and 24 h, while the control group was treated with the corresponding volumes of anhydrous ethanol for the same duration with each treatment, including six well-replicated samples. Cell viability was determined by the CCK-8 assay kit (I-presci scientific); CCK-8 is a commonly used reagent for assessing cell viability and cytotoxicity. For the assay, 10 μL of CCK-8 solution was added to each well, taking care to avoid bubble formation in the wells as it might affect the final OD measurement. The 96-well culture plate was then incubated at 37 °C for 1−4 h.

2.2.7. Cell Staining with Hoechst 33342. First, MAC-T cells were plated in a six-well plate and cultured until they reached confluence of approximately 80% (at approximately 24 h). Then, the cells were treated with a serum-free DMEM medium for 12 h. Subsequently, the cells were treated with varying doses (0, 50, 75, 150, and 300 μmol/L) of *t*10*c*12-CLA for 4, 12, and 24 h; three well-replicated samples were used for all treatments. After each well was rinsed 3 times with normal saline, they were incubated with 1 mL of Hoechst 33342 staining solution (Batch: C0031, Beijing Solarbio Technology Co., Ltd., Beijing, China) and incubated at 37 °C for 30 min. After incubation, the liquid was discarded, and the cells were rinsed 2−3 times with PBS or culture medium. Last, the cells were observed and photographed under a confocal microscope.

2.2.8. Oil Red O Staining. MAC-T cells were cultured in a six-well plate until they reached approximately 80% confluence, after which the growth solution was displaced with serum-free DMEM basal medium and treated for 12 h. Then, the cells were exposed to 300 μM *t*10*c*12-CLA (treatment group) or the corresponding dose of anhydrous ethanol (control group) for 24 h. After cell processing, follow the instructions in the protocol for operation. Last, the cells were observed under a microscope. The results were confirmed based on the data on three biological replicates.

2.2.9. Measurement of Intracellular Triglyceride Levels. MAC-T cells were seeded in a six-well plate and cultured until they reached an approximately 80% confluence. Subsequently, the cells were treated with serum-free DMEM for 12 h. Then, the cells were processed with either 300 μM *t*10*c*12-CLA (treatment group) or the same dose of anhydrous ethanol (control group) for 24 h. Intracellular triglyceride content was determined by a commercial assay kit (Triglyceride Assay Kit; Batch: E1013, Pulead Gene Technology Co., Ltd., Beijing, China). The results were confirmed based on the data from three biological replicates.

2.2.10. Statistics. The Prism10 software was used for analyzing the data (represented as the mean ± standard error) and generating graphs. The differences in parameters between groups were determined by independent samples *t* test using IBM SPSS 27.0. Each experiment was conducted with at least three biological replicates.

3. RESULTS

3.1. Effect of CLA on Milk Yield, Milk Composition, and Total Energy Output during the Perinatal Period. The variations in milk composition, milk yield, and total energy output between the cows in the seven-day postpartum group continuously fed conjugated linoleic acid (CLA) and those in the control group did not show statistical significance. In the 21-day postpartum experimental group, the milk fat percentage decreased on the third day of the experiment, followed by a slow recovery after the end of feeding. During this period, milk protein levels first increased but decreased after feeding was stopped. There were no notable alterations observed in lactose, milk yield, or milk energy output (Figure 1).

3.2. Cell Viability Assay. The proliferation of MAC-T cells after treatment with *t*10*c*12-CLA for 4, 12, and 24 h is

shown in Figure 2. When the concentration of *t*10*c*12-CLA was ≤300 μM, the effect on cell proliferation across different

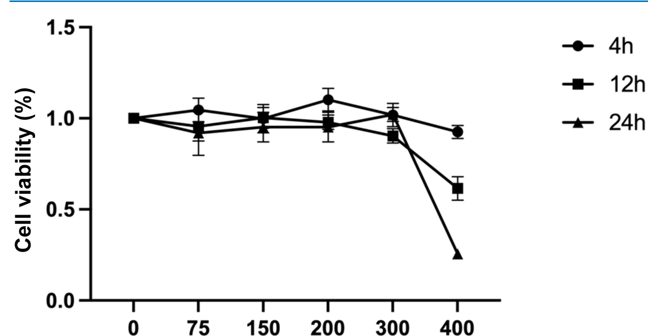


Figure 2. Effect of different concentrations (0, 75, 150, 200, 300, and 400 μM) of *t*10*c*12-CLA on the viability of bovine mammary epithelial cells.

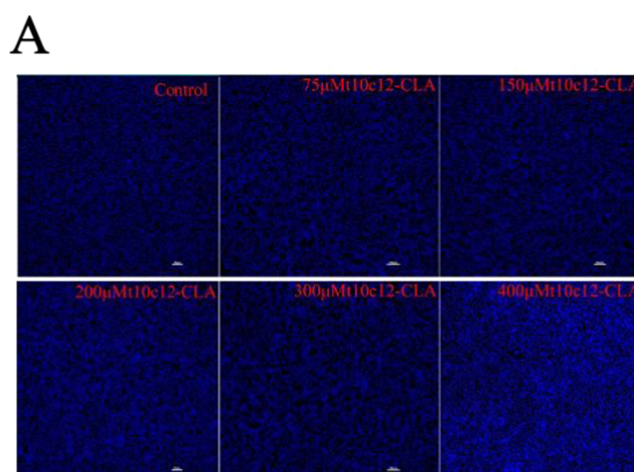
treatment time points was almost unaffected in different treatment time groups, and the cell proliferation in the 4 h treatment group showed an upward trend. However, when the concentration of *t*10*c*12-CLA exceeded 300 μM, cell proliferation was inhibited at 12 h (approximately 30−40%), with significant inhibition observed at 24 h (approximately 70−80%).

3.3. Cell Staining with Hoechst 33342. The growth status of MAC-T cells remained normal when the concentration of *t*10*c*12-CLA was below 400 μM, compared with the cells in the control group, across all three time points. When the concentration of *t*10*c*12-CLA was 400 μM, the growth status of MAC-T cells remained normal after 4 h of treatment. However, after 12 h of treatment, apoptosis was detected in the cultured MAC-T cells, which was characterized by a decrease in cell adhesion and intercellular connectivity, resulting in the formation of gaps. After 24 h of treatment, the number of apoptotic cells raised significantly compared to that recorded after 12 h of treatment along with the presence of numerous gaps and vacuoles (Figure 3).

From the outcomes of the cell proliferation and apoptosis assays, 300 μM *t*10*c*12-CLA was selected as the optimal concentration for subsequent cell treatments. We assessed the level of expression of apoptosis-related genes or proteins in MAC-T cells handled with 300 μM *t*10*c*12-CLA. The level of expression of Bcl2-associated X, apoptosis regulator, B-cell leukemia/lymphoma 2, and cysteinyl aspartate specific proteinase3/9 (Bax, Bcl2, Caspase3, and Caspase9), which are apoptosis-related proteins, was determined (see Figure 4A,B). The outcomes provided evidence that the mRNA levels of expression of Bax and Caspase9 did not show a notable variation between the two groups (Figure 4A; $P > 0.05$); however, the mRNA levels of Bcl2 and Caspase3 were dramatically lower in the *t*10*c*12-CLA-treated group (Figure 4A; $P < 0.01$). The outcomes of the protein assay showed no notable differences in the level of expression of Bax, Bcl2, and Caspase3 (Figure 4B; $P > 0.05$).

3.4. Detection of Oil Red O Staining and Triglyceride Content. As shown in the figure, treatment with 300 μM *t*10*c*12-CLA significantly increased the lipid droplet content in MAC-T cells (Figure 5A), consistent with the lipid droplet effect of palmitic acid (PA), but significantly decreased the intracellular TG content (Figure 5B).

A



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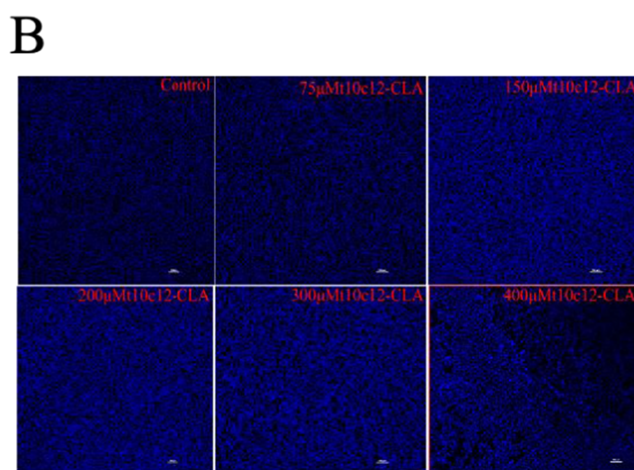

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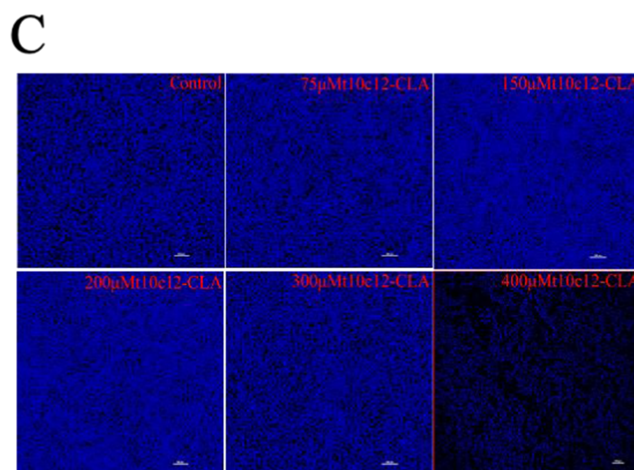

Figure 3. (A) Hoechst 33342 staining of BMECs treated with 0, 75, 150, 200, 300, and 400 μM *t*10*c*12-CLA for 4 h. (B) Hoechst 33342 staining of bovine mammary epithelial cells treated with 0, 75, 150, 200, 300, and 400 μM *t*10*c*12-CLA for 12 h. (C) Hoechst 33342 staining of bovine mammary epithelial cells treated with 0, 75, 150, 200, 300, and 400 μM *t*10*c*12-CLA for 24 h.

3.5. Gene Expression Associated with Lipid Metabolism. The synthesis of milk fat involves processes such as de novo biosynthesis of SCFAs and MCFAs, uptake, transport, and activation of LCFAs, synthesis of TGs, and synthesis and secretion of lipid droplets. To elucidate and investigate the

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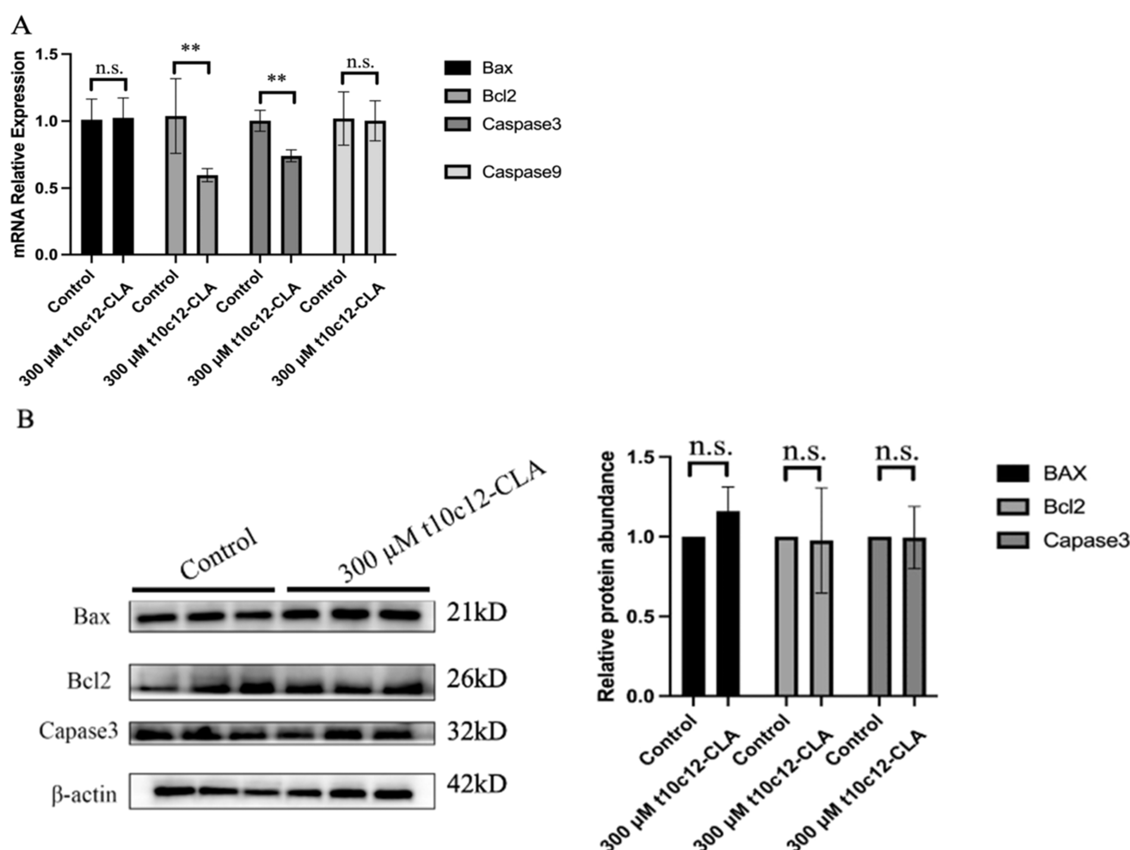

Figure 4. Impact of 300 μM *t*10*c*12-CLA on cell apoptosis. (A) The level of expression of Bax, Bcl2, Caspase3, and Caspase9 mRNAs was corrected using GAPDH. (B) Western blotting analysis of Bax, Bcl2, and Caspase3 in the control group and CLA-treated group. Each data is presented as the mean ± standard error of the mean; * $P < 0.05$ and ** $P < 0.01$.

contradictory relationship between lipid droplet accumulation and a reduction in milk fat levels, we examined the level of expression of the genes or proteins associated with milk fat biosynthesis under the influence of *t*10*c*12-CLA. First, we analyzed the impact of *t*10*c*12-CLA on the de novo biosynthesis of FAs. The results indicated a notable diminution responsible for the de novo biosynthesis of SCFAs and MCFAs (ACACA1 and FASN) in the treated group. The content of the transcription factor SREBP1 also decreased significantly (Figure 6; $P < 0.01$).

Subsequently, the analysis of the genes related to the transport of LCFAs showed that 300 μM *t*10*c*12-CLA significantly downregulated the genes FABP3, ACSL1, and ACSL4 (Figure 7; $P < 0.01$), as well as FATP3 (Figure 7; $P < 0.05$), but it significantly upregulated CD36 (Figure 7; $P < 0.01$).

The catalytic action of AGPAT6, LPIN1, DGAT1, and DGAT2 is essential for the triglyceride synthesis. In this study, treatment with 300 μM *t*10*c*12-CLA led to a notable diminish in the level of expression of the LPIN1 and DGAT1 genes (Figure 8; $P < 0.01$) but a notable raise in the level of expression of the DGAT2 gene (Figure 8; $P < 0.01$); however, it did not significantly affect the expression of the AGPAT6 gene (Figure 8; $P > 0.05$).

More lipid droplets formed after triglyceride synthesis. The examination of the genes associated with lipid droplet formation showed that 300 μM *t*10*c*12-CLA significantly elevated the activity of the ADRP gene but diminished the activity of the XDH gene (Figure 9A; $P < 0.01$); yet the expression of the BTN1A1 gene was unaffected (Figure 9A; $P > 0.05$). Additionally, treatment with 300 μM *t*10*c*12-CLA exhibited effects on the ADRP and XDH protein levels that were consistent with the mRNA expression levels (Figure 9B; $P < 0.01$), but it markedly diminished the level of expression of the BTN1A1 protein (Figure 9B; $P < 0.05$).

The analysis of genes related to lipid membrane fusion showed that treatment with 300 μM *t*10*c*12-CLA significantly decreased the expression of the SNAP23 gene (Figure 10A; $P < 0.01$), while it had no impact on the expression of the VAMP4 gene (Figure 10A; $P > 0.05$). Additionally, treatment with 300 μM *t*10*c*12-CLA markedly reduced the level of expression of the SNAP23 and VAMP4 proteins (Figure 10B; $P < 0.01$).

4. DISCUSSION

4.1. Impact of *t*10*c*12-CLA on Postpartum Milk Composition, Milk Yield, and Milk Net Energy Output in Dairy Cows.

Milk composition is a crucial determinant of dairy product quality and directly affects the profitability of dairy farming. The components of the milk composition in cows primarily include milk fat, lactose, and milk protein. Previous studies mostly assessed the impacts of *t*10*c*12-CLA on milk fat, and only a few studies investigated its effect on lactose, milk protein, and total energy output. The findings of our experiment showed that the milk composition of cows 7 days postpartum was unaffected by *t*10*c*12-CLA, but significant changes in milk composition were observed 21 days postpartum following supplementation with *t*10*c*12-CLA. It is speculated that during the early postpartum period, cows

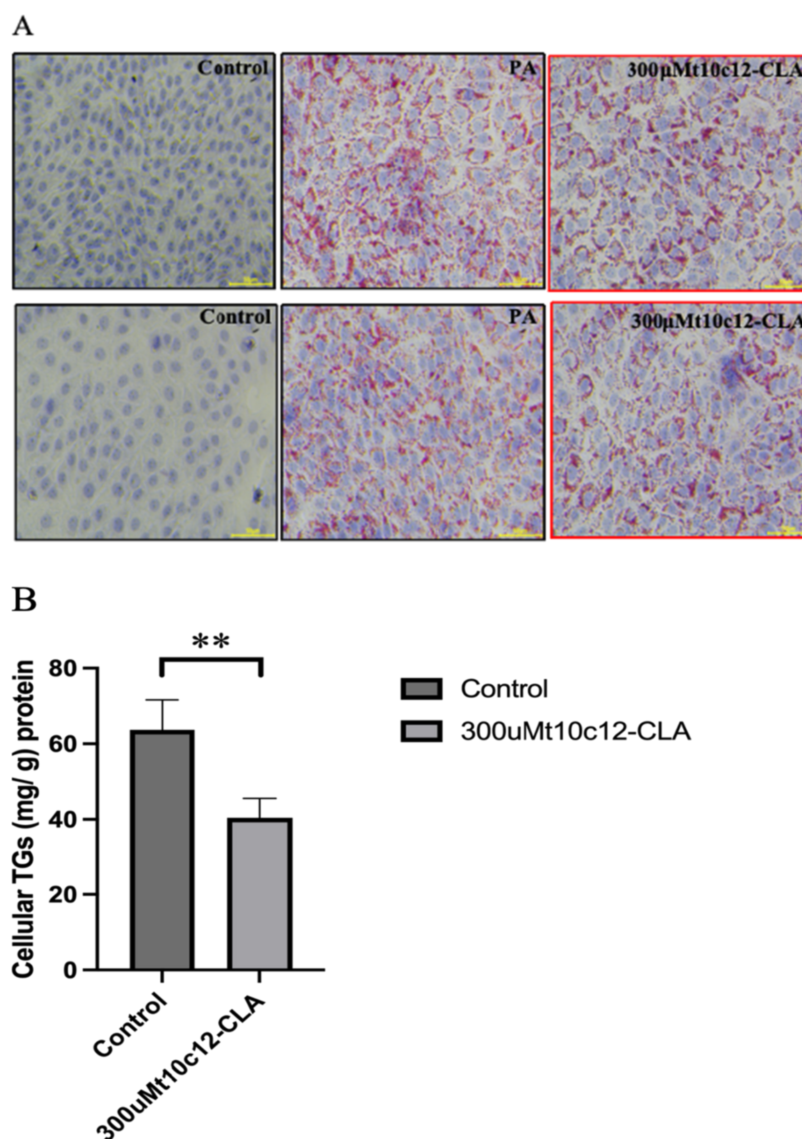

Figure 5. (A) Oil Red O staining of MAC-T cells treated with 300 μM *t*10*c*12-CLA(B). Effect of 300 μM *t*10*c*12-CLA on intracellular triglyceride levels. The outcomes are expressed as the mean ± standard error; "*" $P < 0.05$; "**" $P < 0.01$.

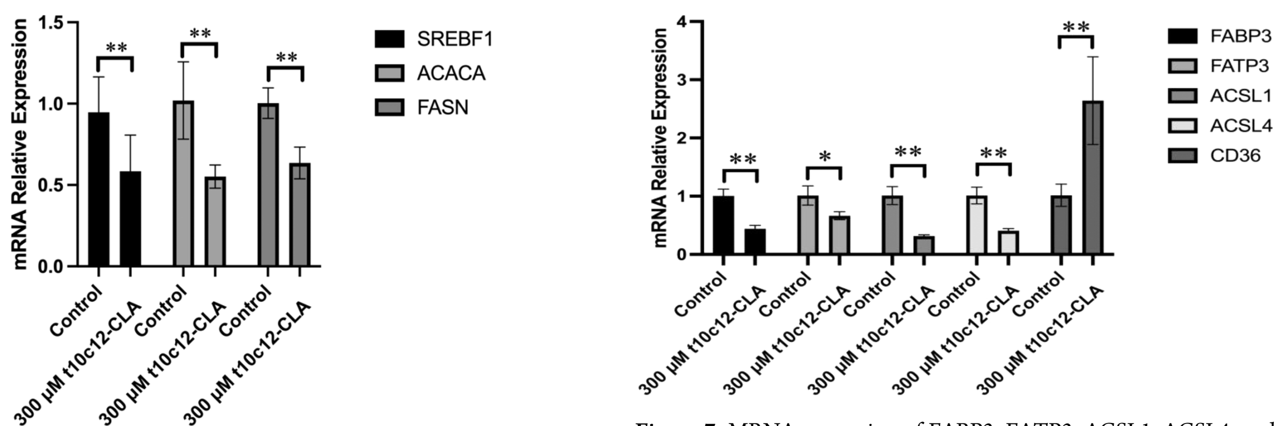

Figure 6. MRNA expression of SREBP1, ACACA1, and FASN in MAC-T with 300 μM *t*10*c*12-CLA. The data are presented as the mean ± standard error; "*" $P < 0.05$ and "**" $P < 0.01$.

Figure 7. MRNA expression of FABP3, FATP3, ACSL1, ACSL4, and CD36 in MAC-T with 300 μM *t*10*c*12-CLA. All data expressed as the mean ± standard error; "*" $P < 0.05$ and "**" $P < 0.01$.

experience high energy demands and mobilization of body fat,[20] which may mask the impacts of *t*10*c*12-CLA on milk fat. With the advancement of the postpartum period and a decrease in fat mobilization, mammary glands may rely more on nutrient intake from feed, allowing the impacts of *t*10*c*12-

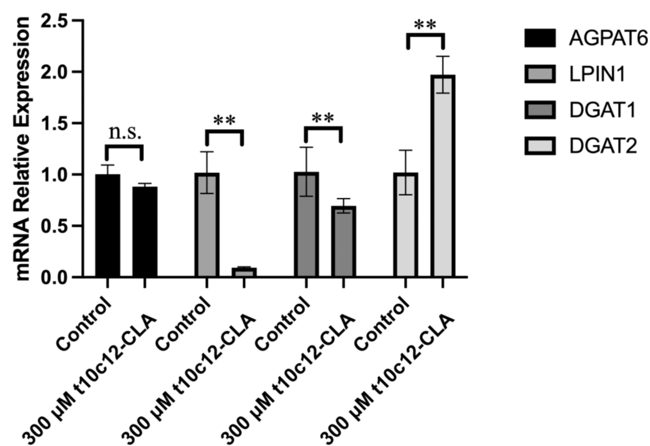

Figure 8. MRNA expression of AGPAT6, LPIN1, DGAT1, and DGAT2 in MAC-T with 300 μM *t*10*c*12-CLA. Each data is expressed as the mean ± standard error of the mean; "*" $P < 0.05$ and "**" $P < 0.01$.

CLA to manifest. Bell and Kennelly found that after intravenous infusion of CLA in cows, milk fat levels decreased along with other adverse effects, including a decrease in lactose and milk yield, although milk protein percentage increased with a decrease in yield.[21] Similarly, de Veth et al. administered different forms of CLA to cows and found that CLA reduced milk fat while not altering milk protein levels or milk yield;[15] similar findings were reported by Baumgard and Michael, indicating that CLA had no impact on milk yield or milk protein, and only *t*10*c*12-CLA supplementation impacted milk fat.[6] Rahbar et al. reported a downgrade in milk fat and a swell in milk production, but no alterations were observed in other properties.[22] Except for the results for milk fat, our other experimental results were different from those reported in these studies. We speculated several reasons for this discrepancy: (1) differences in the composition of CLA mixtures, where different compositions might affect milk composition differently, (2) potential discrepancies in milk composition analysis, leading to inconsistent data across studies, and (3) differences in methods of administration,

Figure 9. Illustration of the impact of 300 μM *t*10*c*12-CLA on lipid droplet synthesis. (A) The level of expression of ADRP, BTN1A1, and XDH mRNAs was normalized using GAPDH as a reference gene. (B) Western blotting analysis of ADRP, BTN1A1, and XDH in the control and CLA-treated groups. Each data is expressed as the mean ± standard error of the mean; "*" $P < 0.05$ and "**" $P < 0.01$.

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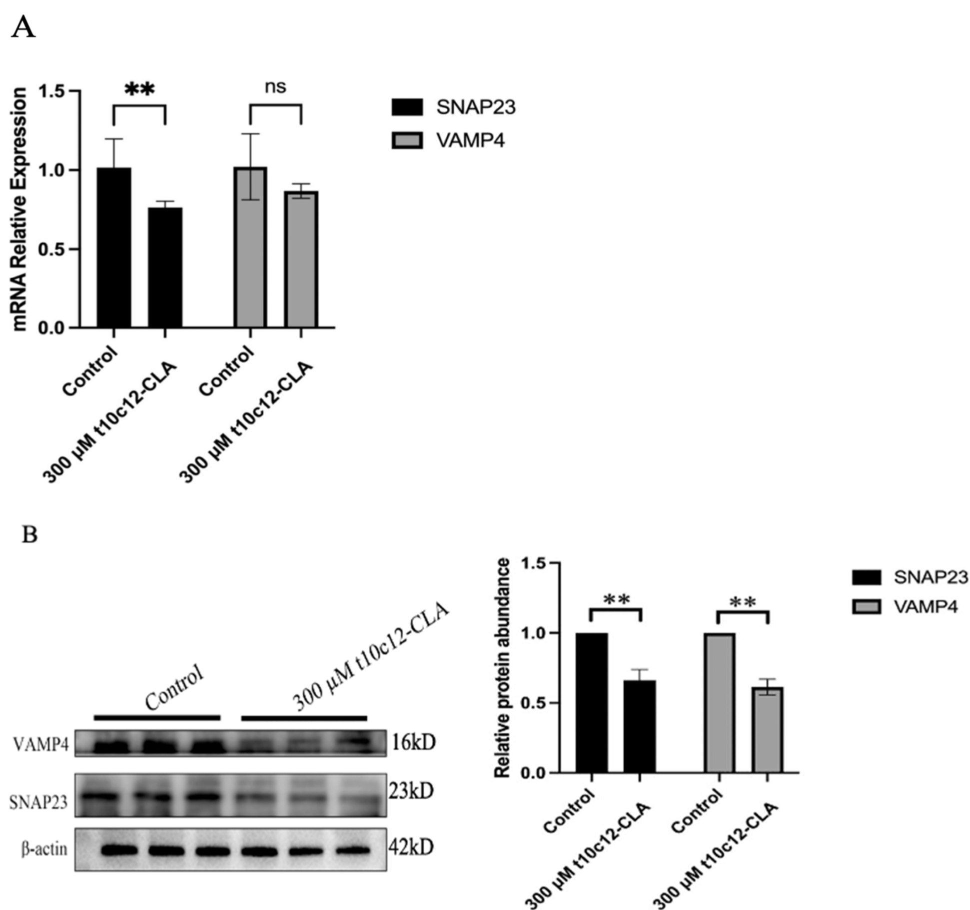

Figure 10. Impact of 300 μM *t*10*c*12-CLA on lipid droplet fusion. (A) GAPDH-normalized level of expression of SNAP23 and VAMP4 mRNAs. (B) Western blotting analysis of SNAP23 and VAMP4 in the control and CLA-treated groups. Each data is expressed as the mean ± standard error of the mean; "*" $P < 0.05$ and "**" $P < 0.01$.

such as intravenous infusion, oral gavage, or abomasal infusion, which may influence the outcomes. In our experiment, CLA supplementation resulted in an increase in milk protein without altering the energy output or milk yield, possibly due to energy savings from milk fat synthesis being redirected toward milk protein synthesis. However, further studies are needed to validate this conclusion. Future studies might include in vitro experiments to establish a model of energy deficit in bovine mammary epithelial cells (using low-glucose culture media) and assess changes in milk protein-synthesis-related pathways at the genetic and protein levels following CLA treatment. Such information can help determine whether CLA can increase milk protein synthesis and confirm the energy-saving effect of milk fat synthesis for producing milk proteins.

A study showed that *t*10*c*12-CLA is the primary trans-fatty acid accountable for the diminution of milk fat in dairy cows.[23] Our findings showed that *t*10*c*12-CLA can reduce milk fat in cows, as reported in many studies. Although many studies have investigated the inhibitive effect of *t*10*c*12-CLA on milk fat, the specific mechanisms by which *t*10*c*12-CLA regulates lipid metabolism in cows are not fully known. Therefore, we used BMECs to further explore the functions by which *t*10*c*12-CLA regulates lipid metabolism.

4.2. Impact of *t*10*c*12-CLA on the Growth of MAC-T.

To select the perfect concentration of *t*10*c*12-CLA for treating MAC-T cells, we conducted cell proliferation and apoptosis experiments. We found that *t*10*c*12-CLA exhibited concentration- and time-dependent effects on the proliferation of MAC-T cells. Low concentrations of *t*10*c*12-CLA and short-term treatment did not inhibit proliferation, whereas high concentrations and long-term treatment inhibited cell proliferation. Eun J Kim stimulated Caco-2 cells with 5 μM *t*10*c*12-CLA for 96 h and found a 55 ± 3% reduction in cell number, demonstrating that *t*10*c*12-CLA reduced IGF-II secretion in a dose-dependent manner to mediate growth inhibition.[24] This finding matched our results, indicating a correlation between the dose of *t*10*c*12-CLA and its effect on cell proliferation. Peterson et al. found that dissimilar concentrations of *t*10*c*12-CLA exert a dose-dependent influence on the expression of lipogenic genes (SREBP1, ACACA1, and FASN) in MAC-T cells, while other genes showed significant changes only at specific concentrations.[1] This finding suggested that concentration assays are demanded to research the regulatory effects of certain genes or proteins in the process of suppression of milk fat biosynthesis by CLA, without any alterations in cell proliferation. The growth stage (proliferation, differentiation, and apoptosis) of MECs influenced remodeling and involution of bovine mammary gland tissue during the periparturient period.[25] The apoptosis of cells is regulated by the endogenous apoptotic pathway through proteins in the B-cell lymphoma 2 protein (Bcl2) family, where antiapoptotic proteins like Bcl2 bind to pro-apoptotic proteins like Bcl2-associated X protein (Bax) to inhibit apoptosis by preventing Bax oligomerization.[26] Excessive Bax activity relative to Bcl2 triggers caspase cascade

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reactions, which in turn are regulated by Caspase9 and Caspase3 in the early and late stages of apoptosis, respectively.[27] Some studies have shown dose-dependent induction of caspase-mediated apoptosis by *t*10*c*12-CLA in MIP-101 and PC-3 cells.[28] 50 μM *t*10*c*12-CLA caused the apoptosis of HCT-116 cells by upregulating pro-apoptotic genes, nonsteroidal anti-inflammatory drug-activated gene 1, and activating transcription factor-3.[29] In dRLH-84 cells of rats, 1 μmol/L *t*10*c*12-CLA was found to induce apoptosis by activating caspases3 and 9.[30] These studies on the mechanisms underlying apoptosis induced by different concentrations of *t*10*c*12-CLA in different cells suggested that *t*10*c*12-CLA may induce apoptosis by regulating Bax, Bcl2, and caspases3 and 9. Our results showed that 300 μmol/L *t*10*c*12-CLA downregulated the mRNA levels of Bcl2 and caspases3, but it did not influence the expression of Bax, Bcl2, or caspases3 proteins, indicating that 300 μmol/L *t*10*c*12-CLA does not affect the proliferation of Mac-T. Cell staining with Hoechst 33342 also showed that 300 μmol/L *t*10*c*12-CLA did not affect the apoptosis of Mac-T. Therefore, without affecting proliferation or apoptosis, we selected 300 μmol/L *t*10*c*12-CLA for subsequent experimentations.

4.3. Impact of *t*10*c*12-CLA on Lipid Accumulation. The impact of dietary factors on milk composition is crucial, where high-concentrate diets can result in MFD. Among various hypotheses proposed regarding MFD, the trans-fatty acid theory, particularly *t*10*c*12-CLA, has gained widespread recognition as an effective inhibitor of milk fat biosynthesis.[31,32] As milk fat is primarily composed of TGs, *t*10*c*12-CLA can inhibit the synthesis of TGs in milk fat. Under normal physiological conditions, TG in cells is synthesized in the endoplasmic reticulum, where the synthesized TAGs aggregate into small lipid droplets. Adjacent droplets merge and enlarge as they move toward the apical membrane, where large lipid droplets are secreted into the alveolar lumen encapsulated by the plasma membrane.[33] Thus, triglyceride accumulation leads to the constitution of more lipid droplets that are subsequently secreted into the milk pool to form milk fat globules. Consistent with the findings of other studies, the outcomes of our in vitro assays showed that treatment with 300 μM *t*10*c*12-CLA significantly reduced the triglyceride content in bovine mammary epithelial cells. Feeding mixed CLA isomers or *t*10*c*12-CLA to humans can reduce body fat levels,[34,35] and treating human adipocyte cultures with *t*10*c*12-CLA can decrease cell size and TG content.[36] Some long-chain fatty acids can act similarly to conjugated linoleic acid. Hansen and Kundone found that adding stearic acid and linoleic acid to cultured MECs inhibited the involvement of free FAs in TG synthesis, thus reducing intracellular triglyceride content.[37] However, many studies reported findings that were different from our experimental results. Chen et al. found that feeding mice with CLA increased intramuscular fat (IMF) deposition by upregulating the level of expression of PPARα and A-FABP mRNAs and proteins in mouse muscle tissue.[38] Gwang-woong Go treated HepG2 cells with 100 μM *t*10*c*12-CLA for 72 h and found that the level of expression of the mRNAs and proteins of lipid source genes such asSREBP1, ACC1, FASN, ELOVL6, GPAT1, and DGAT1 increased, which promoted TG synthesis.[39] Thus, we speculated that the mechanisms underlying the regulatory effects of *t*10*c*12-CLA may vary in different tissues or cells. However, Kadegowda et al. found that adding 18:0, 16:0, and *t*10*c*12-CLA increased the triglyceride content in mammary epithelial cells by 80, 140, and 250%, respectively.[8] Zhang et al. stimulated goat mammary epithelial cells (GMECs) with 100 μM *t*10*c*12-CLA and found that *t*10*c*12-CLA increased TG biosynthesis in vitro in goat mammary epithelial cells (GMECs).[40] Besides conjugated linoleic acid, other LCFAs can stimulate TG biosynthesis.[40] Yonezawa et al. treated BMECs with 50−400 μM different LCFAs and found that all FAs promoted triglyceride synthesis; specifically, a linear correlation was found between the content of TG produced and the concentration of LCFAs.[41] Therefore, *t*10*c*12-CLA may exert different effects on the synthesis of triglycerides in the same tissue or organ, probably due to variations in the concentration of *t*10*c*12-CLA. Thus, the modulation of triglycerides by *t*10*c*12-CLA may produce opposing effects depending on the tissue or organ and the concentration of *t*10*c*12-CLA administered.

The accretion of TG promotes the formation of lipid droplets under normal physiological conditions. Yet, in this study, staining with Oil Red O revealed an addition in the quantity of lipid droplets in BMECs treated with 300 μM *t*10*c*12-CLA. This finding was contradictory to the significant diminution in intracellular triglyceride content observed after treatment with 300 μM *t*10*c*12-CLA. To explore the mechanism by which *t*10*c*12-CLA decreases triglyceride synthesis but increases the quantity of lipid droplets in BMECs, we examined typical genes interrelated with the intake, transport, activation of LCFAs, de novo biosynthesis of MCFAs and SCFAs, TGs, and lipid droplet formation and secretion. Many studies have investigated the regulatory impact of *t*10*c*12-CLA on the de novo biosynthesis of FAs. For example, Lin et al. demonstrated that CLA isomers (*t*10*c*12-CLA) can reduce fat production in lactating mouse mammary glands, probably by decreasing enzyme activity and the level of ACC mRNAs.[42] Robblee et al. demonstrated that *t*10*c*12 CLA represses milk fat synthesis by reducing FASN activity.[43] Baumgard et al. fed cows with 3.5, 7.0, and 14.0 g/day of *t*10*c*12-CLA, which produced a diminution in milk fat by 24, 37, and 46%, respectively. They also determined the formation of FAs in milk and found that 7.0 and 14.0 g/d of *t*10*c*12-CLA substantially reduced de novo FAs biosynthesis (SCFAs and MCFAs).[5] ACACA and FASN play crucial roles in de novo biosynthesis of FAs.[44,45] ACACA is the rate-limiting step in FAs de novo biosynthesis and catalyzes the conversion of acetyl-CoA to malonyl-CoA for biosynthesizing SCFAs, MCFAs, and palmitic acid.[11] FASN is primarily responsible for catalyzing the biosynthesis of long-chain fatty acyl-CoA from acetyl-CoA and malonyl-CoA, but it can also catalyze the production of short-chain, medium-chain fatty, and very long-chain fatty acids using specific substrates.[46] SREBP1 is a central transcription factor regulating the synthesis of milk fats, mainly related to the synthesis of FAs and TGs, and it exerts a direct regulatory effect on a variety of pathways interrelated with lipid metabolism.[11] Bidault et al. showed that SREBF1, as a major regulatory factor, can regulate de novo biosynthesis of FAs by promoting the expressions of ACACA and FASN.[47] In this study, 300 μM *t*10*c*12-CLA significantly downregulated the mRNA levels of SREBP1 and SREBF1 in MAC-T cells. This is consistent with the Research Report of Chen et al.[48] The mRNA levels of ACACA and FASN also showed a significant reduction, which indicated that ACACA1 and FASN are target genes of the transcription factor SREBF1. To summarize, the findings of in vitro and in vivo assays manifested that *t*10*c*12-CLA can downregulate the synthesis

of MCFAs and SCFAs mediated by ACACA1 and FASN by reducing the levels of SREBF1.

These findings indicated that the increase in lipid droplets is probably not attributed to an increase in the biosynthesis of MCFAs and SCFAs. Next, we investigated the intake, transport, and activation of LCFAs. LCFAs are in the extracellular environment and can enter cells in two ways: (1) They can be actively absorbed by mammary epithelial cells through FAs translocase (CD36/SR-B2) and FAs transport proteins (SLC27A-SLC27A6/FATP). CD36/SR-B2 recruits extracellular FAs to the surface of the cell membrane, which are then transferred to FATPs for transport.[49] (2) LCFAs may be absorbed by MECs via a flip-flop mechanism. LCFAs enter MECs through the aforementioned pathways, forming long-chain acyl-CoA through the activation of ACSL1.[50] Finally, cytoplasmic FABPc binds to released LCFAs and transports them to the target organelles. Long-chain-CoA is transported to specific cells after binding to FABP3. Bionaz and Loor found that the level of expression of CD36 mRNA increased markedly during lactation in cows, indicating its effect on the synthesis of milk fats.[11] They also showed that during lactation, FABP3 expression in the mammary glands of cows was the highest. FABP3 could also bind to oleic acid, stearic acid, and palmitic acid and function cooperatively with ACSL1 and SLC27A6 to esterify LCFAs into triglycerides in milk. We speculated that the increase in lipid droplets may be due to the uptake and utilization of *t*10*c*12-CLA as LCFAs by MECs. Kadegowda et al. found that certain concentrations of C18:0, C20:5, and *t*10*c*12-CLA significantly increased the level of expression of CD36.[8] Zhang et al. reported that 100 μM *t*10*c*12-CLA significantly raised the level of CD36 mRNA in GMECs.[40] Chen et al. found that LCFAs can enhance CD36 activity yet reduce FABP3 expression.[51] Our outcomes also showed a notable increase in the expression of CD36 mRNA, which explained the origin of lipid droplets. However, the level of expression of FATP, ACSL1, ACSL4, and FABP3 mRNAs decreased by different degrees, which contradicted the previous conclusion. Therefore, further studies are needed on exogenous *t*10*c*12-CLA as a source of lipid droplets. Peterson et al. found that feeding cows with a high-concentrate diet containing *t*10*c*12-CLA led to a diminution in the expression of FABP mRNA, which showed an inverse correlation with the concentration of *t*10*c*12-CLA in the diet;[52] this finding also matched the in vitro results of our study.

Lipid droplets originate due to the accumulation of triglycerides, and triglyceride synthesis is regulated by three enzymes, including AGPAT6, LPIN1, and DGAT. Batista et al. found that in bovine embryo cells, *t*10*c*12-CLA can reduce lipid content by decreasing the expression of 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) genes.[53] Sørensen et al. found that various CLA isomers (including *t*10*c*12-CLA) decreased DGAT enzyme activity, but they did not affect DGAT1 while increasing the abundance of DGAT2 mRNAs.[54] In animal experiments, Vyas et al. found that feeding lactating cows with 10 g/d of *t*10*c*12-CLA downregulated the abundance of the mRNAs of triglyceride synthesis-related enzymes AGPAT6 and DGAT1.[55] Our experimental results showed that 300 μM *t*10*c*12- CLA induced a remarkable diminish in the activity of LPIN1 and DGAT1, while markedly increasing the expression of DGAT2, with no effect on AGPAT6. Our experimental results regarding AGPAT6 were different from previous research findings. Kadegowda et al. found that by adding different long-chain fatty acids to MAC-T cells, 100 μM *t*10*c*12-CLA markedly elevated the expression of DGAT1 and AGPAT6 mRNAs but downregulated the expression of LPIN1. However, in their study, 100 μM *t*10*c*12-CLA led to the accumulation of triglycerides in cells.[8] We speculated that these differences in results might be due to the different concentrations of *t*10*c*12-CLA. Although low concentrations of *t*10*c*12-CLA can inhibit lipid synthesis, it may be used as a substrate to promote triglyceride synthesis; thus, *t*10*c*12-CLA can mitigate the inhibitory effect on triglyceride synthesis. At high concentrations, the inhibitory effect of *t*10*c*12-CLA outweighs its role as a substrate for triglyceride synthesis. Our outcomes demonstrated that DGAT2 was significantly upregulated, which may represent a pathway for *t*10*c*12-CLA to serve as a substrate for triglyceride synthesis, and lipid droplets may originate from this pathway.

Lipid droplet formation is reported by Walther et al.[56] In this section, we describe the relevant genes related to lipid droplet genesis. BTN1A1, ADRP (PLIN), and XDH participate in lipid droplet genesis. In MECs, the trans-membrane lipid transport protein BTN1A1 regulates the quantity and size of lipid droplets.[57] XDH (XOR) mainly packages lipid droplets at the apex of the membrane. The perilipins (PLINs) that are predominantly located at the surface of lipid droplets are PLIN1 and PLIN2. As the lipid droplets enlarge, PLIN2 is replaced by PLIN1. PLIN1 prevents adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) from entering lipid droplets to interact with triglycerides.[58,59] Our study's results demonstrated that 300 μM *t*10*c*12-CLA significantly upregulated ADRP (PLIN1) and downregulated the expression of XDH mRNA, but the degree of upregulation of ADRP exceeded the downregulation of XDH. The abundance of the BTN1A1 mRNA was not affected by 300 μM *t*10*c*12 CLA, but the expression of the BTN1A1 protein reduced markedly. Although several studies have extensively investigated the formation of lipid droplets, the study of the impact of *t*10*c*12-CLA on lipid droplets is limited. Zhang et al. found that treating goat mammary epithelial cells with 100 μM *t*10*c*12-CLA significantly increased ADRP (PLIN1) and decreased the expression of XDH mRNA; additionally, the upregulation of ADRP greatly exceeded the downregulation of XDH.[40] Our findings were similar to those of the above-mentioned study. Therefore, we speculated that *t*10*c*12-CLA can promote lipid droplet genesis in MAC-T cells, possibly through ADRP as the primary regulatory gene.

We speculated that lipid droplets increased because *t*10*c*12-CLA (a LCFAs) provides raw materials for the formation of triglycerides and is regulated by ADRP. Zhang et al. analyzed the FAs composition of goat milk after feeding *t*10*c*12-CLA to goats and observed a remarkable raise in the proportion of C18:2 FAs.[60] This finding also provided support for *t*10*c*12-CLA as a long-chain fatty acid for triglyceride formation. Lipid droplets fuse to create larger droplets, which are then secreted into the milk pool to form milk fat globules (milk fat). As *t*10*c*12-CLA suppresses milk fat production in dairy cows, the reason behind an increase in lipid droplets with a diminution in milk fat is worth further exploration. We studied the fusion and secretion of the lipid droplets. There are two hypotheses concerning the mechanism of lipid droplet secretion to form milk fat globules: (1) Lipid droplets are encased by the cell membrane at the apex of MECs, forming milk fat globule membranes containing three layers of membranes. (2) Lipid droplets fuse to create larger droplets, which are then discharged by exocytosis.[61−63] The milk fat globule membrane

(MFGM) contains eight kinds of proteins, with the abundance of BTN1A1 and XDH being the highest.[64] XDH and BTN are involved in the process of enveloping lipid droplets and the subsequent secretion at the outer membrane of mammary gland cells.[65,66] Our results showed that 300 μM *t*10*c*12-CLA markedly restrained the activity of XDH but did not influence the activity of BTN1A1. However, 300 μM *t*10*c*12-CLA markedly decreased the protein expression of XDH and BTN1A1. This indicated that *t*10*c*12-CLA not only inhibits triglyceride and medium-chain FAs biosynthesis but also inhibits the extracellular secretion of lipid droplets. Lipid droplet fusion is also regulated by the fusion mediator SNARE proteins. Boström et al. markedly diminished the fusion rate and size of lipid droplets by knocking down the genes of SNAP23, syntaxin-5, or VAMP4 in SNARE proteins.[14] Therefore, SNARE may participate in the fusion of lipid droplets. Fu et al. determined that SNAP23 positively regulates the fusion of lipid droplets.[67] Other studies have not observed an influence of *t*10*c*12-CLA on SNARE proteins. Our results showed that 300 μM *t*10*c*12-CLA markedly decreased the level of expression of SNAP23 mRNA but did not affect VAMP4 mRNA levels. To summarize, *t*10*c*12-CLA not only inhibits the extracellular secretion of lipid droplets but also inhibits the fusion of lipid droplets.

5. CONCLUSIONS

Our experiments provided new insights into the influences of *t*10*c*12-CLA on postpartum dairy cow milk composition, milk yield, milk net energy, and lipid metabolism in MECs. When 136.17 g of RP-CLA was administered to dairy cows, it did not affect the production performance, milk composition, and net energy of lactation of dairy cows at 7 days postpartum, but it diminished milk fat percentage and raised milk protein percentage in cows without affecting milk yield and milk net energy on day 21 postpartum. The energy saved from milk fat, which constitutes the most energy-consuming component in milk, may be used for milk protein synthesis, although additional experiments are required to validate this postulate. Administering 300 μM *t*10*c*12-CLA repressed the activity of genes concerned with de novo FAs biosynthesis (SREBP1, FASN, and ACACA), the uptake, transport, and activation of LCFAs (FABP3, FAT3, ACSL1, and ACSL4), triglyceride biosynthesis (DGAT1 and LPIN1), lipid droplet constitution (BTN1A1 and XDH), and lipid droplet fusion and secretion (BTN1A1, XDH, SNAP23, and VAMP4), but it increased the activity of DGAT2, CD36, and ADRP (PLIN1). We proposed that lipid droplets may originate from *t*10*c*12-CLA, as it can be activated through uptake to form lipid droplets. An upregulation of the expression of CD36, DGAT2, and ADRP (PLIN1) may provide support for this hypothesis. A reduction in milk fat in MAC-T cells, coupled with an enhancement in cytoplasmic lipid droplets, appears to be contradictory. By studying lipid droplet fusion and extracellular secretion, we found that 300 μM *t*10*c*12-CLA inhibited lipid droplet fusion and extracellular secretion, thus reducing the production of milk fat globules (milk fat).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c05532.

Composition table of conjugated linoleic acid mixture; and primers used for real-time quantitative PCR (PDF)

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Notes

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■ ABBREVIATIONS

ACACA1, acetyl-CoA carboxylase; ACSL1, acyl-CoA synthetase long-chain family member 1; ACSL4, acyl-CoA synthetase long-chain family member 4; AGPAT6, 1-acylglycerol-3-phosphate O-acyltransferase 6; ADRP/PLIN2, perilipin2; ACSS2, acetyl-CoA synthetase short-chain family member 2; ATGL, adipose triglyceride lipase; Bax, Bcl2-associated X, apoptosis regulator; Bcl2, B-cell leukemia/lymphoma 2; BTN1A1, butyrophilin subfamily 1 member A1; BSA, bovine serum albumin; BCS, body condition score; CD36, thrombospondin receptor; CLA, conjugated linoleic acid; Caspase3/9, cysteinyl aspartate specific proteinase3/9; DGAT1/2, diacylglycerolacyl transferase1/2; FASN, fatty acid synthase; FABP3, fatty acid-binding protein 3; FATP3/SLC27A3, solute carrier family 27 member 3; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSL, hormone-sensitive lipase; INSIG1, insulin-induced gene 1; LPIN1, lipin 1; LCFAs, long-chain fatty acids; MAC-T, mammary alveolar cells-large T; MFD, milk fat depression; SNAP23, synaptosome associated protein 23; SCD, stearoyl-CoA desaturase; *t*10*c*12-CLA, *trans*-10, *cis*-12 conjugated linoleic acid; TMR, total mixed ration; TAG/TG, triglycerides; THRSP, thyroid hormone-inducible hepatic protein; VAMP4, vesicle-associated membrane protein 4; XDH, xanthine dehydrogenase

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