Milk fat globule membrane proteins are involved in controlling the size of milk fat globules during conjugated linoleic acid–induced milk fat depression

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ABSTRACT

Milk fat globule membrane (MFGM) proteins surround the triacylglycerol core comprising milk fat globules (MFG). We previously detected a decrease in the size of fat globules during conjugated linoleic acid (CLA)-induced milk fat depression (MFD), and other studies have reported that some MFGM proteins play a central role in regulating mammary cellular lipid droplet size. However, little is known about the relationship between MFD, MFG size, and MFGM proteins in bovine milk. The aim of this study was to investigate the profile of MFGM proteins during MFD induced by CLA. Sixteen mid-lactating Holstein cows (145 ± 24 d in milk) with similar body condition and parity were divided into control and CLA groups over a 10-d period. Cows were fed a basal diet (control, n = 8) or control plus 15 g/kg of dry matter (DM) CLA (n = 8) to induce MFD. Cow performance, milk composition, and MFG size were measured daily. On d 10, MFGM proteins were extracted and identified by quantitative proteomic analysis, and western blotting was used to verify a subset of the identified MFGM proteins. Compared with controls, supplemental CLA did not affect milk production, DM intake, or milk protein and lactose contents. However, CLA reduced milk fat content (3.73 g/100 mL vs. 2.47 g/100 mL) and the size parameters volume-related diameter D[4,3] (3.72 μm vs. 3.35 μm) and surface area-related diameter D[3,2] (3.13 μm vs. 2.80 μm), but increased specific surface area of MFG (1,905 m2/kg vs. 2,188 m2/kg). In total, 177 differentially expressed proteins were detected in milk from cows with CLA-induced MFD, 60 of which were upregulated and 117 downregulated. Correlation analysis showed that MFG size was negatively correlated with various proteins, including XDH and FABP3, and positively correlated with MFG-E8, RAB19, and APOA1. The results provide evidence for an important role of MFGM proteins in regulating MFG diameter, and they facilitate a mechanistic understanding of diet-induced MFD.

Key words: milk fat depression, milk fat globule, milk fat globule membrane protein, milk fat globule size

INTRODUCTION

Diet-induced milk fat depression (MFD) in dairy cattle is characterized by a reduction in milk fat content with no change in milk yield or levels of other milk components (Bauman and Grimari, 2000). Addition of CLA can have a specific inhibitory effect on the content of milk fat in dairy cows (Baumgard et al., 2001; Giesy et al., 2002; Bauman and Grimari, 2003). Only a limited number of mechanisms have been investigated in CLA-induced MFD, mostly at the level of gene expression (Bernard et al., 2008; Han et al., 2012; Harvatine et al., 2018). Recently, it was reported that the decrease in milk fat content is accompanied by a reduction in milk fat globule (MFG) size (Xing et al., 2020), suggesting that mechanisms controlling MFG size are a component of MFD.

In milk, phospholipids and proteins wrap around triglycerides to form MFG (Lönnerdal, 2016). The precursor of MFG are lipid droplets (LD), with small LD fusing to form larger LD before being released from mammary epithelial cells into milk in the form of MFG (McManaman et al., 2007). The normal secretion of small and large LD results in a wide range in the diameter of MFG (0.1–15 μm) in milk. Lipid droplets are coated by lipid-protein membranes derived from the plasma membrane (Jeong et al., 2013).
Two main components on the surface of LD, phospholipids and proteins, are thought to control LD size (Co- hen et al., 2015). Proteomic analysis revealed that the overall protein composition of LD isolated from mouse mammary cells is similar to that of MFG obtained from milk (Wu et al., 2000). In cellular and mouse models, there is evidence that proteins on the surface of LD are a key factor controlling their size. For instance, abundance of ADPH correlates with cytoplasmic lipid drop-let size and accumulation in mouse mammary glands (Russell et al., 2007). Because LD are the precursors of MFG, MFG membrane (MFGM) proteins are clearly involved in MFG formation, but the specific proteins involved in ruminants remain unknown.

We hypothesized that the abundance of MFGM proteins may be associated with the size of secreted MFG. Thus, we reasoned that detailed proteomics analysis of MFGM proteins in MFG of different sizes would help identify the proteins contributing to the regulation of MFG size and further explain the mechanisms associated with MFD in ruminants. The objective of the present work was to investigate the relationships between the size of MFG and MFGM proteins resulting from CLA-induced MFD in bovine milk. To our knowledge, this is the first study to use proteomics technology to discern changes in MFGM proteins in response to diet-induced MFD in ruminants.

**MATERIALS AND METHODS**

**Animals**

All procedures involving cows were in accordance with the ethical standards of the Animal Care Commit- tee of Henan Agricultural University (Henan Province, China). All animals received humane care according to Management of Animal Care and Use Programs in Research, Education, and Testing, 2nd ed (Weichbrod et al., 2018). Sixteen healthy mid-lactation Holstein cows (145 ± 24 DIM, 554 ± 31.4 kg of BW, 26.8 ± 2.7 kg of milk/d) were selected and randomly divided into control and CLA groups for a 10-d feeding study in a dairy farm located in Zhengzhou City (Henan Province, China). The control group (n = 8) was fed a basal diet and the CLA group (n = 8) was fed the control diet plus Ca-protected CLA at 15 g/kg DM (Qing-dao Auhai Biotech Co. Ltd.). The basal diet (Table 1) was prepared according to the guidelines for dairy cows in NRC (2001). The commercial rumen-protected CLA contained 38.1% cis-9,trans-11 and 36.8% trans-10,cis-12 isomers (Table 2), and this was added to the TMR together with concentrate, fully mixed, and fed to cows.

Dairy cows were housed in the same 2-row barn with individual tiestalls to reduce environmental interfer- ence, and they had ad libitum access to food and drinking water. Cows were milked 3 times a day at 0600, 1400, and 2000 h, and fed a TMR after milking at 0600 and 1400 h. Daily milk production and DMI were re- corded for each cow. Milk samples were collected once a day at the 1400 h milking and divided into 2 parts; one sample was sent to the DHI Testing Center of Henan Province (Zhengzhou City, China), and milk composi- tion was determined using infrared spectrophotometry using a Foss 120 MilkoScan instrument (Foss Electric); the second sample was immediately transported to

**Table 1.** Ingredients and chemical composition (g/kg DM unless otherwise noted) of the basal diets of cows

| Composition       | Content |
|-------------------|---------|
| Ingredient        |         |
| Corn silage       | 420     |
| Ground corn       | 49      |
| Steam-flaked corn | 147     |
| Soybean meal      | 85      |
| Cottonseed        | 40      |
| Oat hay           | 42      |
| Alfalfa hay       | 132     |
| Soybean hull      | 56      |
| Yeast XP1         | 2.2     |
| NaCl              | 3.8     |
| Limestone         | 4.8     |
| NaHCO3            | 8.2     |
| CaHCO3            | 3.4     |
| MgO               | 1.6     |
| Premix2           | 5       |
| Chemical composition |     |
| CP                | 167     |
| NDF               | 315     |
| ADF               | 208     |
| Ether extract     | 56      |
| Net energy of diet | 1.71  |
| Ca                | 8.1     |
| P                 | 4.5     |

1Purchased from Diamond V Co.

2Composition per kg of DM: 650,000 IU of vitamin A; 92,000 IU of vitamin D3; 3,750 IU of vitamin E; 700 mg of niacin; 2,000 mg of Cu; 4,000 mg of Zn; 330 mg of Mn; 12 mg of Se; 65 mg of I; 37 mg of Co.

3Calculated using NRC (2001).

**Table 2.** Fatty acid composition of Ca-CLA fed to Holstein cows to induce milk fat depression (n = 8)

| Fatty acid | % of total fatty acids |
|-----------|-----------------------|
| C16:0     | 6.2                   |
| C18:0     | 2.0                   |
| C18:1     | 10.6                  |
| C18:2 cis-9,cis-12 | 1.6     |
| CLA       | 79.6                  |
| C18:2 cis-9,trans-11 | 38.1   |
| C18:2 trans-10,cis-12 | 36.8   |
| Other CLA isomers | 4.6     |
| Others    | <0.1                  |

discern changes in MFGM proteins in response to diet-induced MFD in ruminants.
the laboratory and used to measure the particle size of MFG with a Mastersizer 3000 laser particle size analyzer (Malvern Ltd.). On d 10 of treatment, milk samples were collected and MFGM proteins extracted and stored at −80°C for proteomics analysis.

**MFG Size Parameters**

A laser particle size analyzer was used to detect the size of MFG in fresh milk samples. Briefly, the laser refractive index was set to pure water = 1.330 and milk = 1.560. Water (400 mL) was added to a beaker, stirred at 2,000 × g for 1 min, and the background was measured before adding 1 mL of milk sample to the beaker until the laser intensity indicated 10% to 20%. Mastersizer software was used for measurements to generate data including volume-related diameter (D[3,2]), surface area-related diameter (D[a,2]), and specific surface area (SSA).

**Extraction of MFGM Proteins**

Proteins were extracted as described previously (Yang et al., 2016; Manoni et al., 2020) with modifications. Briefly, fresh milk samples were centrifuged for 30 min at 3,000 × g and 4°C to harvest the fat layer; an equal volume of deionized water was then added into the cream, and the mixture stirred slowly in a 40°C water bath for 10 min. The mixture was then centrifuged for 15 min at 3,500 × g and 25°C and the fat cream layer isolated. Ultrasonication of creams was performed for 5 min to break MFG, until oily substances precipitated. The mixture was then stirred slowly in a 40°C water bath for 10 min followed by centrifugation for 15 min at 3,500 × g and 25°C. After collecting the floating fat layer and incubating for 12 h at 4°C, cream was melted in a 40°C water bath, an equal volume of cracking solution (dithiothreitol:Tris-HCl:SDS, 15:1:60) was added, and samples were incubated at room temperature for 60 min, and then centrifuged for 20 min at 8,000 × g and 25°C. After collecting the pellet, precooled acetone was added, and samples were incubated for 10 to 15 h at −20°C before being centrifuged for 20 min at 8,000 × g and 25°C. Then, the supernatant was discarded and the MFGM protein pellet was air-dried and dissolved in 0.1 M HCl. The obtained MFGM proteins were stored at −80°C for further analysis.

**SDS-PAGE for Identification of MFGM Proteins**

The MFGM protein samples (10 μg) were mixed with 0.5 mL of reducing buffer (6% Tris-0.5 M, 10% glycerol, 5% β-mercaptoethanol, 2% SDS, 0.05% bromophenol blue) and heated in boiling water for 3 min. After centrifugation for 10 min at 12,000 × g (4°C), the supernatant was separated by 12% SDS-PAGE at 80 V for 30 min, followed by 120 V for 120 min. After electrophoresis, the gel was stained and destained for 10 min, and images were scanned using an Amersham Imager 600 instrument (GE Healthcare).

**Sample Digestion and Labeling for Quantitative Proteomic Analysis**

Samples (100 μg) were placed in a 1.5-mL centrifuge tube. A 1:20 trypsin enzyme: substrate protein solution was added, vortexed, centrifuged at 1,000 × g, 4°C, for 1 min, and incubated for 4 h at 37°C. The digested peptide solution was desalted and freeze-dried. After returning the solution to room temperature, 50 μL of isopropanol was added to each tube and vortexed at low speed. The peptide sample was dissolved in 0.5 M tetraethylammonium bromide and added to the corresponding iTRAQ (isobaric tags for relative and absolute quantitation) labeling reagent.

**Peptide Fractionation**

A Shimadzu LC-20AB liquid-phase system equipped with a 5-μm, 4.6- × 250-mm Gemini C18 column was used for liquid-phase separation of samples. Dried peptide samples were reconstituted with mobile phase A consisting of 5% acetonitrile (ACN; pH 9.8) and injected into the instrument. The elution flow rate was 1 mL/min, and the gradient was 5% mobile phase B (95% ACN, pH 9.8) for 10 min; 5 to 35% mobile phase B for 40 min; 35 to 95% mobile phase B for 1 min; mobile phase B for 3 min; and 5% mobile phase B for 10 min. Elution peaks were monitored at a wavelength of 214 nm, and 1 fraction was collected per minute. Samples were combined according to the chromatographic elution peak profile, and fractions were freeze-dried.

**HPLC-Tandem Mass Spectrometry**

Dried peptide samples were reconstituted with mobile phase A, comprising 2% ACN and 0.1% formic acid, centrifuged for 10 min at 20,000 × g (4°C), and the supernatant was collected for injection. Separation was performed using a Thermo UltiMate 3000 UHPLC instrument (Thermo Fisher Scientific). Samples were first enriched using a trap column, desalted, and then separated in a self-packed C18 column (75 μm internal diameter, 3 μm column size, 25 cm column length) at a flow rate of 300 nL/min with the following gradient: 0 to 5 min, 5% mobile phase B (98% ACN, 0.1% formic acid); 5 to 45 min, mobile phase B linearly increased from 5 to 25%; 45 to 50 min, mobile phase B increased to 100%.
from 25 to 35%; 50 to 52 min, mobile phase B increased from 35 to 80%; 52 to 54 min, 80% mobile phase B; 54 to 60 min, 5% mobile phase B. The nanoliter liquid-phase separation column was directly connected to the mass spectrometer.

Peptides separated by liquid-phase chromatography were ionized with an electrospray ionization (ESI) source and then passed on to a Q-Ex active HF X tandem mass spectrometer (Thermo Fisher Scientific) for data-dependent acquisition mode detection. The main parameters were ion source voltage 1.9 kV, MS1 scanning range 350–1,500 m/z, resolution 60,000, MS2 starting m/z fixed at 100, and resolution 15,000. Ion screening conditions for MS2 fragmentation were charge 2+ to 6+, and the top 20 parent ions with a peak intensity >10,000. Ion fragmentation mode was high-energy collision dissociation, and fragment ions were detected in an Orbitrap Mass Analyzer (Thermo Fisher Scientific). Dynamic exclusion time was set to 30 s, and automatic gain control for full MS target and MS2 starting m/z was filtered at the 1% false discovery rate at the peptide level. Milk fat globule membrane proteins with fold change >1 and P < 0.05 were considered upregulated; those with fold change <1 and P < 0.05 were considered downregulated. Correlations between proteins and MFG size parameters were assessed using mstools software (https://mstools.shinyapps.io/shiny/).

RESULTS

Western Blotting

According to previous research on LD proteins in mammary cells (Robenek et al., 2006; Chong et al., 2011) and MFGM proteins in milk (Liao et al., 2011; Li et al., 2019), xanthine dehydrogenase (XDH), butyrophilin (BTN), and perilipin2 (PLIN2) were selected to verify the reliability of proteomics results because they are the most abundant MFGM proteins, and they are involved in regulating LD size. Sixty micrograms of extracted MFGM protein per lane was separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% skim milk for 2 h, membranes were incubated with primary antibody overnight at 4°C. Primary antibodies used in this study were anti-tubulin (1:1,000, ab179513, Abcam), anti-BTN1A1 (1:50, BM5509, Acris), anti-PLIN2 (1:500, 15294, Proteintech), and anti-XDH (1:500, 55156, Proteintech). After washing 5 times with Tris-buffered saline with Tween (TBST), membranes were probed with horseradish peroxidase-conjugated AffiniPure Goat anti-rabbit/anti-mouse IgG (H+L); 1:2,000, Proteintech) for 2 h at room temperature. Quantification of immunoblots was performed using the standard enhanced chemiluminescence procedure (Amersham ECL Prime, GE Healthcare) and Image J software (National Institutes of Health).

Statistical Analysis

Milk yield and composition and MFG size were analyzed using the general linear model of SPSS 24 (IBM Corp.) for a full factorial model with repeated measures. The model included time, treatment, and time × treatment interactions as fixed effects, and cow as a random effect. Results are reported as mean ± standard error of the mean. Proteomics data were converted from the original HPLC-tandem MS (HPLC-MS/MS) search for data using Mascot (version 2.3.02; http://www.matrixscience.com/mascot_support.html) against the Bos taurus MFGM proteins database (https://www.ncbi.nlm.nih.gov/genome/?term=bos+taurus). Quantitative analysis of peptides was performed using iQuant software (Wen et al., 2014). Results were filtered at the 1% false discovery rate at the peptide level. Milk fat globule membrane proteins with fold change >1 and P < 0.05 were considered upregulated; those with fold change <1 and P < 0.05 were considered downregulated. Correlations between proteins and MFG size parameters were assessed using mstools software (https://mstools.shinyapps.io/shiny/).

Effect of CLA on Lactation Performance, Milk Composition, and MFG Size

As shown in Table 3, no differences (P > 0.05) were observed between treatments for cow milk yield, DMI, or milk protein and lactose contents, whereas a significant reduction in milk fat content (P < 0.01) was observed. Compared with controls, MFG in the CLA group tended to have a lower average D_{4,3} (3.72 μm vs. 3.35 μm; P < 0.01) and lower D_{3,2} (3.13 μm vs. 2.80 μm; P < 0.01, Table 3). Feeding CLA led to higher SSA (1,905 m^2/kg vs. 2,188 m^2/kg; P < 0.01).

SDS-PAGE Analysis of Identified MFGM Proteins

Total proteins from milk and MFGM proteins isolated from fresh bovine milk were analyzed by SDS-PAGE (Figure 1). Compared with total milk proteins, following extraction, MFGM proteins such as XDH, BTN, and PLIN2 were enriched, whereas casein content was greatly reduced.

Proteomic Analysis

In the present study, 177 differentially expressed proteins were detected in cow milk with CLA-induced MFD, of which 60 (including SERPINB5, MUC15, VLDLR, and MFG-E8) were upregulated and 117 (including RPS28, FABP3, XDH, and PLIN2) were downregulated in milk from cows fed CLA (Table 4). For XDH and PLIN2, western blotting results were similar to proteomics results, with expression decreased
by 0.86- and 0.74-fold, respectively; no significant difference was observed for BTN (Figure 2, \(P > 0.05\)).

**Correlation Analysis**

Correlations among MFG size parameters \(D_{4,3}\), \(D_{3,2}\), SSA, and MFGM protein expression are shown in Figure 3A–C). Diameter \(D_{4,3}\) and SSA were negatively correlated with a variety of proteins, including XDH, NAPA, FABP3, TLN1, PLSCR2, TWF1, STK, YES1, C1B1, GDI2, LOC781001, STXBP2, RAB18, ENKUR, BCAS3, ENKUR, RAB9B, FOCD, AHCYL2, SNX12, and NECTIN2, but positively correlated with MFG-E8, APOA1, APOC3, RAB19, LGALS8, ERLIN2, C9, CCT3, ARPC2, EEF3B, CRP, CIS, CCT4, LOC100297192, DCTN2, GLOD4, GMPPA, CHMP2B, CAPNS1, IGLL1, RPL27A, RPL29, SH3GLB2, NPEPPS, TCAF1, ERLEC1, HSPA5, ACTR1A, and SPINT2. Diameter \(D_{3,2}\) and SSA were positively correlated with ALDH1A1, EIF5, and EIF3F, and negatively correlated with TWF1, NPC2, and BCAS3. Size measurements \(D_{4,3}\), \(D_{3,2}\), and SSA were positively correlated with RAB19, GLOD4, and RPL27A, but negatively correlated with ENKUR.

**DISCUSSION**

In recent decades, trans-fatty acid theory has been proposed to explain MFD, but the specific mechanism is not clear. It is well known that supplementation of CLA reduces the content of milk fat, resulting in MFD. The inhibitory effect of CLA on the de novo synthesis of milk fat in ruminants has been verified at both the cellular and nutritional levels (Mu et al., 2021). The mRNA abundance of key fatty acid synthesis enzymes (e.g., FASN, ACC, SREBP1) is downregulated by CLA supplementation in ruminants (Gervais et al., 2009; Han et al., 2012). Harvatine et al. (2018) found no effect of CLA on MFGM-associated genes, including BTN1A1, PLIN2, and XDH. In the present study, addition of Ca-CLA caused a substantial decline in milk fat (Table 3). At the same time, we found that CLA supplementation significantly reduced the diameter of MFG, as shown previously (Xing et al., 2020). These results confirm that supplementation of CLA reduces

| Item | Treatment | SEM | Treatment | Time | Treatment × time |
|------|-----------|-----|-----------|------|-----------------|
| DMI (kg/d) | Control | 16.74 | 15.88 | 0.44 | 0.20 | 0.62 | 0.60 |
| Milk yield (kg/d) | Control | 30.23 | 29.59 | 0.92 | 0.36 | 0.28 | 0.68 |
| Milk fat (g/100 mL) | Control | 3.73 | 2.47 | 0.21 | <0.01 | 0.01 | 0.03 |
| Milk protein (g/100 mL) | Control | 3.53 | 3.29 | 0.05 | 0.09 | 0.05 | 0.54 |
| Milk lactose (g/100 mL) | Control | 5.08 | 5.16 | 0.04 | 0.38 | 0.06 | 0.81 |
| \(D_{4,3}\) (μm) | Control | 3.72 | 3.35 | 0.06 | <0.01 | <0.01 | 0.01 |
| \(D_{3,2}\) (μm) | Control | 3.13 | 2.80 | 0.05 | <0.01 | <0.01 | <0.01 |
| SSA (m²/kg) | Control | 1,905 | 2,188 | 40.91 | <0.01 | <0.01 | <0.01 |

1\(D_{4,3}\) = volume mean diameter; \(D_{3,2}\) = surface area mean diameter; SSA = specific surface area.
2\(n = 8\) cows in control and CLA groups. Control cows were fed a basic diet for 10 d, and CLA cows were fed the control diet plus CLA at 15 g/kg DM for 10 d.
fat content and MFG size in dairy cows. Proteomic analysis identified 60 proteins that were upregulated and 117 proteins that were downregulated, some of which, including XDH, FABP3, and MFG-E8, were significantly correlated with MFG diameter. We showed for the first time that CLA supplementation affected the expression of MFGM proteins associated with MFD and a reduction in MFG diameter.

Diameter of MFG can be determined before and during LD secretion from mammary epithelial cells; hence, studies on the size regulation of LD can reveal the mechanisms controlling MFG size (Argov-Argaman, 2019). Lipid droplets originate from the endoplasmic reticulum in the cytoplasm, and are surrounded by polar lipids and proteins from the endoplasmic reticulum. After LD are transported to the cytoplasm, the small LD fuse or increase in volume, become surrounded by the apical plasma membrane, and are then secreted into milk as MFG. Therefore, MFGM proteins are composed of proteins from both the cytoplasm and the plasma membrane. Of the proteins identified in the bovine MFGM, 71% are membrane associated and 29% are cytoplasmic or secretory (Brink and Lönnerdal, 2020). The size of LD is affected by many factors in mammary cells, including triglyceride synthesis, phospholipids and proteins in the membrane, and LD fusion and interaction with organelles (Argov-Argaman, 2019). Advances in quantitative proteomics methods

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Table 4. Effects of Ca-CLA supplementation on relative milk fat globule membrane (MFGM) protein expression levels in dairy cows (n = 3)

| Protein   | Up/down | Protein   | Up/down | Protein   | Up/down | Protein   | Up/down |
|-----------|---------|-----------|---------|-----------|---------|-----------|---------|
| RPS28     | ↓       | EIF5      | ↓       | MBL2      | ↓       | SELENBP1  | ↑       |
| RAB9B     | ↓       | ENKUR     | ↓       | MFGES     | ↓       | SELENOF   | ↑       |
| ACTR1A    | ↓       | ERLC1     | ↓       | MOCS1     | ↓       | SERPINA1  | ↑       |
| ADGRF1    | ↑       | ERLIN2    | ↓       | MOG       | ↓       | SERPINB5  | ↑       |
| AHCYL2    | ↓       | F2        | ↓       | MUC15     | ↓       | SERPIN1   | ↑       |
| ALDH1A1   | ↓       | FABP3     | ↓       | MYB7      | ↓       | SIBBP4    | ↑       |
| ALOX15     | ↑       | FGR       | ↓       | NAPA      | ↓       | SH3GL2    | ↑       |
| ANKRD22   | ↓       | FKBPI1    | ▲       | NECTIN2   | ↓       | SH3G4L2   | ↑       |
| APOA1     | ↓       | FLOT2     | ↓       | NPC2      | ↓       | SH3Y1L    | ↑       |
| APOC3     | ↓       | FOAD       | ↓       | NPEP5     | ↓       | SLC3A10   | ↑       |
| ARF2      | ▲       | FOLR2     | ↓       | NTSE      | ↓       | SLC7A2    | ↑       |
| ARPC2     | ↓       | FOLR3     | ↓       | NUCB1     | ↓       | SNAP29    | ↑       |
| BCS3      | ↓       | GALE      | ↓       | OSTF1     | ↓       | SNX1      | ↓       |
| BRK1      | ▲       | GDI2      | ↓       | PAAH1B3   | ↓       | SNX2      | ↓       |
| C1S       | ▲       | GFRA2     | ↓       | PANX1     | ↓       | SPINT2    | ↑       |
| C3        | ▲       | GIPG2     | ↓       | PDC1D0    | ↓       | SQLE      | ↑       |
| C9        | ▲       | GIPG2     | ↓       | PFN1      | ↓       | STK38L    | ↑       |
| CAB39      | ▲       | GLOD4     | ↓       | PGK1      | ↓       | STX9      | ↑       |
| CAPNS1     | ▲       | GLRM      | ↓       | PIGN      | ↓       | STXBP2    | ↑       |
| CARHSP1   | ▲       | GLYCAM1   | ↓       | PLN2      | ↓       | STXBP6    | ↑       |
| CCDC47    | ▲       | GMPPA     | ↓       | PLSCR2    | ↓       | TAGLN2    | ↑       |
| CCT3      | ▲       | GOLPH3L   | ↓       | PPA1      | ↑       | TCAF1     | ↓       |
| CCT4      | ▲       | GUK1      | ↓       | PUDP      | ↓       | TFL1      | ↓       |
| CD5L       | ▲       | HSPA1A    | ↓       | PXN       | ↓       | TLR2      | ↓       |
| CD9       | ▲       | HSPA5     | ↓       | RAB18     | ↓       | TMC4      | ↓       |
| CFB       | ▲       | IGL1      | ↓       | RAB19     | ↓       | TMEM263   | ↓       |
| CF1       | ▲       | IGLL1     | ↓       | RAB27A    | ↓       | TOLEL     | ↓       |
| CHMP2B     | ▲       | KRT10     | ↓       | RAB35C    | ↓       | TOM1      | ↓       |
| CHMP3      | ▲       | LASP1     | ↓       | RAC1      | ↓       | TOM1L1    | ↓       |
| CIB1       | ▲       | LAGLS1    | ↓       | RALB      | ↓       | TPM3      | ↓       |
| CLEC3B     | ▲       | LAGLS8    | ↓       | RAP2C     | ↓       | TRG1L     | ↓       |
| CNNM3     | ▲       | LIN7C     | ↓       | RHEB      | ↓       | TSTA3     | ↓       |
| CREG1      | ▲       | LMAN1     | ↓       | RHOG      | ↓       | TWF1      | ↓       |
| CRP        | ▲       | LOC100297192 | ↓  | RIT1      | ↓       | TXNDC12   | ↑       |
| CTSL       | ▲       | LOC100297193 | ↓  | RNAE4     | ↓       | TXN1L     | ↓       |
| DARS       | ▲       | LOC588579 | ↓       | RPL24     | ↓       | UBA1      | ↓       |
| DCTN2      | ▲       | LOC768101 | ↓       | RPL27A    | ↓       | UFLC1     | ↑       |
| DTO2D      | ▲       | LOC783680 | ↓       | RPL29     | ↓       | VAT1      | ↓       |
| DYN1C1H1   | ▲       | LRRCC8C   | ↓       | RPS5      | ↓       | VCL       | ↑       |
| EIF3B      | ▲       | LSS       | ↓       | RPS9      | ↓       | VLDLR     | ↓       |
| EIF3F      | ▲       | LTF       | ↓       | SAA4      | ↓       | VPS37B    | ↓       |
| EIF4A1     | ▲       | LYPLA2    | ↓       | SDF4      | ↓       | XDH       | ↓       |

↓ = downregulated; ▲ = upregulated.
have facilitated the characterization of MFGM proteins. Although MFGM proteins account for only 1 to 4% of total milk proteins, >500 proteins with diverse functions have been identified by proteomics analysis (Silva et al., 2021), and human, bovine, and donkey MFGM proteins have been investigated (Liao et al., 2011; Li et al., 2019; Manoni et al., 2020). The major bovine MFGM proteins include XDH, PLIN2, BTN, FABP, MUC1, and MUC15. In the present study, in both control and CLA groups, the MFGM protein extraction process was sufficient to remove most serum proteins such as caseins, and enrich MFGM proteins such as XDH, BTN, and PLIN2 (Figure 1). A total of 177 differentially expressed proteins were identified in milk from cows with CLA-induced MFD, of which 60 were upregulated (including MFG-E8, MUC15, and VLDLR) and 117 were downregulated (including XDH, PLIN2, and FABP3). Among these, some are known to play an important role in regulating LD size in cells (Lee et al., 2018), including XDH and PLIN2, but the functions of most have not yet been elucidated, including MFG-E8 and FABP3.

A structural model has been proposed to explain how interactions of XDH with other MFGM proteins, including BTN and PLIN2, may regulate milk fat droplet secretion in lactating mammary epithelial cells in mice (McManaman et al., 2002; Jeong et al., 2013). Xanthine dehydrogenase is a soluble cytosolic enzyme mainly concentrated in the inner leaflet of the plasma membrane in mammary cells. Knockout of the XDH gene in mice resulted in very large LD enclosed in mammary cells (Monks et al., 2016). Butyrophilin is a type I transmembrane glycoprotein that mainly exists in the apical plasma membrane of mammary epithelial cells. As the major protein component of the bovine MFGM, BTN accounts for 20 to 40% of total membrane proteins (D’Alessandro et al., 2010). Perilipin 2 (also named ADPH/ADRP), a member of the perilipin family of LD proteins, is believed to play a key role in the formation and secretion of milk LD (Chong et al., 2011). In the current study, we found that XDH and PLIN2 were significantly decreased according to both proteomics and western blotting data (Table 4 and Figure 2). No difference in BTN was found in the MFGM, although we speculate that BTN regulates the size of LD. Correlation analysis indicated a significant negative correlation between XDH expression and MFG size (D[4,3] and SSA). These results indicate that XDH, one of the most abundant proteins on the membrane of MFG, may be important for regulating the size of fat globules.

Milk fat globule-epidermal growth factor 8 (MFG-E8), also known as lactadherin or PAS-6/PAS-7, is present on the surface of the LD phospholipid bilayer membrane on the apical side of the mammary gland epithelium. Milk fat globule-epidermal growth factor 8 has been linked to membrane vesicle secretion, specifically budding of LD (Oshima et al., 2002). A SNP of MFG-E8 is associated with milk fat yield (Qu et al., 2011). Mice deficient in MFG-E8 form abnormally large LD in the mammary alveolar lumen (Yasueda et al., 2015). In the present study, we found that MFG-E8 was increased in CLA group milk, and correlation analysis showed that expression of MFG-E8 was significantly positively correlated with D[4,3] and SSA (P < 0.01, Figure 3C), which suggests that MFG-E8 in the
lactating mammary gland is crucial for the secretion of milk lipids, especially in the form of small MFG (Sabha et al., 2018).

Fatty acid-binding protein (FABP), comprising several lipid chaperone proteins, plays an important role in long-chain fatty acid uptake, transport, maintenance of cellular lipid homeostasis, and regulation of fat storage (Mu et al., 2021). As a target gene of peroxisome proliferator-activated receptor (PPAR), overexpression of PPAR increases mRNA expression levels of FABP3 and short-chain fatty acids, as well as LD accumulation, by affecting the expression of FABP3 in bovine

Figure 3. Relationships between milk fat globule membrane (MFGM) proteins and milk fat globule size parameters volume mean diameter (D₄₃), surface area mean diameter (D₃₂), and specific surface area (SSA) in CLA-induced milk fat depression. Different colors (pink, green, grey) indicate the strength of correlations: *P < 0.05; **P < 0.01.
mammary epithelial cells (Sun et al., 2016; Shi et al., 2018). In the present study, FABP3 was downregulated in CLA milk and negatively correlated with MFG size, which suggests that FABP3 is a key transcription factor regulating the milk fat globule synthesis signaling pathway (Liang et al., 2014; Mu et al., 2021).

Several cytoplasmic proteins specifically correlated with MFG size were identified in CLA milk. For example, RAB19 (Ras-related protein 19), APOA1 (apolipoprotein A-I preproprotein), ENKUR (enkurin), and EIF5 (eukaryotic translation initiation factor 5) showed positive correlations with MFG size (Figure 3). As a

**Figure 3 (Continued).** Relationships between milk fat globule membrane (MFGM) proteins and milk fat globule size parameters volume mean diameter \((D_{4,3})\), surface area mean diameter \((D_{3,2})\), and specific surface area (SSA) in CLA-induced milk fat depression. Different colors (pink, green, grey) indicate the strength of correlations: *\(P < 0.05\); **\(P < 0.01\).
cytoplasmic protein, RAB19 influences LD interactions with other organelles such as endosomes, peroxisomes, and mitochondria (Li and Yu, 2016). Apolipoprotein A-I is an acceptor of cellular cholesterol secreted from mammary epithelial cells, and it plays a role in influencing milk composition and directing cholesterol back into the bloodstream (Ontsouka et al., 2013). Enkurin is thought to act as an intracellular adaptor protein linking membrane calcium influx to intracellular signaling pathways (Ma et al., 2019), and EIF5 plays an essential role in initiation of protein synthesis in eukaryotic cells (Hinnebusch, 2014). The roles of these proteins...
in regulating LD size is unclear, and further studies are needed. Moreover, how cells regulate membrane proteins during LD fusion remains poorly understood; it is also unclear what happens to MFGM proteins when LD are enveloped by the plasma membrane and secreted into milk to form fat globules. The current results were obtained by analyzing the composition of milk fat globular membrane proteins; hence, as regulatory proteins in mammary epithelial cells, the MFGM proteins (e.g., XDH, MFG-E8) can also be regarded as characteristic of MFG of a specific size in milk caused by CLA. A detailed analysis of these components of MFG could help reveal the underlying mechanisms.

CONCLUSIONS

In the present study, supplementation with CLA during milk production decreased the size of MFG and changed the protein composition of the MFGM. The iTRAQ-labeled proteomics experiment identified 177 significantly differentially expressed MFGM proteins, of which 60 were upregulated and 117 downregulated. Correlation analysis showed that expression of XDH and FABP3 was negatively correlated with MFG size, whereas expression of MFG-E8 and APOA1 was positively correlated with MFG size. The results imply a more complex model for MFG secretion than the current model, and the findings provide insights into the mechanism of MFD.

ACKNOWLEDGMENTS

This research was jointly supported by the National Natural Science Foundation of China (Beijing; U1904116). We are grateful for assistance from Zeng Wang at the Beijing Genomics Institution (Beijing, China) for proteomic experiments and data analysis. The authors have not stated any conflicts of interest.

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