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## Goat $\alpha_{s1}$ -casein genotype affects milk fat globule physicochemical properties and the composition of the milk fat globule membrane

C. Cebo,\*<sup>1</sup> C. Lopez,† C. Henry,‡ C. Beauvallet,\*§ O. Ménard,† C. Bevilacqua,\*§ F. Bouvier,# H. Caillat,|| and P. Martin\*§

\*INRA, UMR1313 Unité Génétique Animale et Biologie Intégrative, F-78350 Jouy-en-Josas, France

†INRA, UMR1253 Science et Technologie du Lait et de l'Œuf (STLO), F-35000 Rennes, France

‡INRA, UMR 1319 MICALIS, PAPSSO (Plateforme d'Analyse Protéomique Paris Sud Ouest), F-78350 Jouy-en-Josas, France

§INRA-Plateforme ICE (Iso Cell Express), F-78350 Jouy-en-Josas, France

#INRA, UE332 Domaine de Bourges, F-18390 Osmoy, France

||INRA, UR631 Station d'Amélioration Génétique des Animaux, F-31426 Castanet-Tolosan, France

### ABSTRACT

Milk fat secretion is a complex process that initiates in the endoplasmic reticulum of the mammary epithelial cell by the budding of lipid droplets. Lipid droplets are finally released as fat globules in milk enveloped by the apical plasma membrane of the mammary epithelial cell. The milk fat globule membrane (MFGM) thus comprises membrane-specific proteins and polar lipids (glycerophospholipids and sphingolipids) surrounding a core of neutral lipids (mainly triacylglycerols and cholesterol esters). We have recently described major proteins of the MFGM in the goat and we have highlighted prominent differences between goats and bovine species, especially regarding lactadherin, a major MFGM protein. Here, we show that, in the goat species, the well-documented genetic polymorphism at the  $\alpha_{s1}$ -casein (*CSN1S1*) locus affects both structure and composition of milk fat globules. We first evidenced that both milk fat globule size and  $\zeta$ -potential are related to the  $\alpha_{s1}$ -casein genotype. At midlactation, goats displaying strong genotypes for  $\alpha_{s1}$ -casein (A/A goats) produce larger fat globules than goats with a null genotype at the *CSN1S1* locus (O/O goats). A linear relationship ( $R^2 = 0.75$ ) between fat content (g/kg) in the milk and diameter of fat globules ( $\mu\text{m}$ ) was established. Moreover, we found significant differences with regard to MFGM composition (including both polar lipids and MFGM proteins) from goats with extreme genotype at the *CSN1S1* locus. At midlactation, the amount of polar lipids is significantly higher in the MFGM from goats with null genotypes for  $\alpha_{s1}$ -casein

(O/O goats;  $5.97 \pm 0.11$  mg/g of fat; mean  $\pm$  standard deviation) than in the MFGM from goats with strong genotypes for  $\alpha_{s1}$ -casein (A/A goats;  $3.96 \pm 0.12$  mg/g of fat; mean  $\pm$  standard deviation). Two MFGM-associated proteins, namely lactadherin and stomatin, are also significantly upregulated in the MFGM from goats with null genotype for  $\alpha_{s1}$ -casein at early lactation. Our findings are discussed with regard to techno-functional properties and nutritional value of goat milk. In addition, the genetic polymorphism in the goat species appears to be a tool to provide clues to the lipid secretion pathways in the mammary epithelial cell.

**Key words:** milk fat globule, goat,  $\alpha_{s1}$ -casein genotype

### INTRODUCTION

Fat is present in milk as droplets of triacylglycerols surrounded by a complex biological membrane deriving from the mammary epithelial cell (MEC) and called the milk fat globule membrane (MFGM). The MFGM has a tripartite structure comprising a monolayer membrane of polar lipids and proteins derived from the endoplasmic reticulum (ER) surrounded by a bilayer membrane of polar lipids containing highly glycosylated proteins arising from the apical plasma membrane of the MEC. Hence, composition of the MFGM likely reflects those of ER and MEC membranes. The primary function of the MFGM is to protect the triacylglycerol core from coalescence and enzymatic degradation by lipases. However, many nutritional benefits have been attributed to both lipid and protein components of the MFGM (Spitsberg, 2005; Dewettinck et al., 2008).

Major MFGM proteins have been extensively reviewed (Mather, 2000). Large-scale proteomic studies on MFGM proteins have also been reported for bovine species (Reinhardt and Lippolis, 2006), humans (Hettinga et al., 2011), and ovine species (Pisanu et al.,

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<sup>1</sup>Corresponding author: christelle.cebo@jouy.inra.fr

2011). Within species, MFGM composition has been shown to be altered by lactation stage (Reinhardt and Lippolis, 2008), diet (Lopez et al., 2008), or even sanitary status of the mammary gland, as mastitis has been recently shown to induce changes in MFGM composition in sheep (Addis et al., 2011). However, to date, no data are reported on the influence of genetic polymorphism occurring within milk protein genes on MFGM composition.

Indeed, in the goat, an extensive genetic polymorphism has been described at the  $\alpha_{s1}$ -CN-encoding gene (*CSN1S1*) with the existence of at least 18  $\alpha_{s1}$ -CN variants, including null alleles. This genetic polymorphism is associated with strong differences in milk protein content. Four levels of  $\alpha_{s1}$ -CN synthesis have been described, ranging between 3.5 and 0 g/L for strong alleles (A, B, and C) and null alleles (O), respectively (Grosclaude et al., 1994; Martin et al., 2002). Moreover, in animals deficient at the *CSN1S1* locus, secretory pathways are severely affected. In the absence of  $\alpha_{s1}$ -CN, an accumulation of immature proteins (mainly caseins) is observed, leading to a dramatic distension of the rough ER (Chanat et al., 1999). An unexpected association of the *CSN1S1* genotype with milk fat concentration has also been reported (Barbieri et al., 1995). The first evidence of the effect of this genetic polymorphism on milk fat secretion arises from transcriptomic studies on mammary tissues. The expression levels of key lipogenic genes as well as genes known to influence membrane fluidity, cell-cell interaction, or chromatin organization are affected by the  $\alpha_{s1}$ -CN genetic polymorphism in the goat (Ollier et al., 2008). Moreover, a study comparing FA profiles in goats displaying strong (A, B, and C alleles), or weak (F and G alleles) genotypes at the *CSN1S1* locus demonstrated differences in proportions in milk fat of 17 FA, including the C8 to C12 SFA and palmitic, stearic, oleic, linoleic, and rumenic acids. In addition,  $\Delta^9$ -desaturated FA were found in higher proportions in the milk from goats with weak genotype for the  $\alpha_{s1}$ -CN gene (Chilliard et al., 2006). These results suggest that genetic polymorphism at the  $\alpha_{s1}$ -CN locus affects not only quantitatively, but also qualitatively, fat in goat milk. However, to date, no data are reported about the effect of  $\alpha_{s1}$ -CN genetic polymorphism on MFGM overall composition.

We, therefore, provide first characterization of milk fat globules in goats with extreme genotypes at the *CSN1S1* locus by combining biophysical and proteomic tools. First, we demonstrate that the  $\alpha_{s1}$ -CN genotype affects the  $\zeta$ -potential of milk fat globules and their size, which is related to the amount of fat in the milk. We also report here that the polar lipid composition of the MFGM is affected by the genetic polymorphism at the

*CSN1S1* locus. Proteomic investigations on the MFGM from goats with extreme genotype for the  $\alpha_{s1}$ -CN gene also demonstrated remodeling of the MFGM from goats with null genotype for  $\alpha_{s1}$ -CN. Taking together, our findings established a link between the  $\alpha_{s1}$ -CN genetic polymorphism, the milk fat content, and the milk fat globule size and composition in goats.

## MATERIALS AND METHODS

### Animals and Milk Samples

Individual milk samples (evening + morning milking) were collected from A/A (animals homozygous for  $\alpha_{s1}$ -CN, allele A) and O/O (animals homozygous for  $\alpha_{s1}$ -CN, allele O,  $\alpha_{s1}$ -CN null animals) primiparous goats at early ( $26 \pm 9$  d postpartum; mean  $\pm$  SD), mid ( $149 \pm 17$  d postpartum; mean  $\pm$  SD), and late ( $250 \pm 9$  d postpartum; mean  $\pm$  SD) lactation stages reared in the Institut National de la Recherche Agronomique (INRA) Domaine de Galle experimental farm (18520, Avord, France). Preservatives [0.05 g of potassium dichromate/L, 10 mM  $\epsilon$ -amino caproic acid, and 10  $\mu$ M phenylmethylsulfonyl fluoride (PMSF)] were added to milk immediately after milking to prevent proteolysis. Milk samples were stored at  $-20^\circ\text{C}$  in 50-mL aliquots except for fat globule size and  $\zeta$ -potential measurements as well as for MFGM polar lipid composition for which nonrefrigerated milk samples were used. After 30-min incubation at  $37^\circ\text{C}$ , milk samples were centrifuged at  $2,000 \times g$  for 15 min at  $20^\circ\text{C}$  and then cooled 30 min at  $4^\circ\text{C}$  to help skimming. Fat globules were recovered in the supernatant layer and washed 3 times with 0.9% (wt/vol) NaCl buffer to remove residual caseins and whey proteins eventually adsorbed to fat globules.

### Fat Globule Size and $\zeta$ -Potential Measurements

The fat globule size distribution was measured by laser light scattering using a Mastersizer 2000 laser diffraction particle size analyzer (Malvern Instruments Ltd., Malvern, Worcestershire, UK) with 2 laser sources. The refractive indexes used were 1.458 and 1.460 at 633 and 466 nm, respectively, and 1.333 for water. Milk samples were diluted in 100 mL of water directly in the measurement cell of the apparatus to reach 10% obscuration. Standard parameters were calculated by Mastersizer 2000 software (Malvern Instruments Ltd.): the modal diameter ( $D_{\text{mode}}$ ), which corresponds to the population of fat globules the most important in volume, and the specific surface area  $S = 6\varphi/d_{32}$ , where  $\varphi$  is the volume fraction of milk fat and  $d_{32}$ , defined as  $\Sigma n_i d_i^3 / \Sigma n_i d_i^2$ , is the volume-surface average diameter,

where  $n_i$  is the number of fat globules of diameter  $d_i$ . Three successive measurements in the absence or in the presence of EDTA (to dissociate casein micelles) were performed for each milk sample. The  $\zeta$ -potential of milk fat globules was calculated from their electrophoretic mobility, using a Zetasizer 3000HS (Malvern Instruments Ltd.) by applying a 25 V/cm voltage at 25°C. For the measurements, 3  $\mu$ L of milk sample was diluted in 10 mL of buffer (20 mM imidazole, 50 mM NaCl, and 5 mM  $\text{CaCl}_2$ , pH 7.0) to mimic milk ionic strength. Analysis of variance was performed using the general linear model procedure of Statgraphics Plus version 5 (Statistical Graphics Corp., Englewood Cliffs, NJ). Differences between the treatment means were compared at the 5% level of significance using Fisher's least significance difference (LSD) test.

### **Extraction and Analysis of MFGM Polar Lipids**

An adapted protocol of the cold extraction procedure developed by (Folch et al., 1957) was used for the extraction of total lipids from goat milk, as detailed in Lopez et al. (2008). Total lipid extracts were stored at  $-20^\circ\text{C}$  until further analysis by HPLC. The quantification of total polar lipids [e.g., glycerophospholipids and sphingomyelin (SM)] and the determination of the polar lipid classes were performed using HPLC combined with an evaporative light-scattering detector (ELSD). The chromatographic method used for the separation of the polar lipids was detailed in Lopez et al. (2008). The identification of the glycerophospholipids and the SM was carried out by a comparison with the retention time of pure standards (supplied by Sigma-Aldrich, Saint Quentin Fallavier, France). To obtain a quantitative evaluation of the glycerophospholipids and SM, 5 calibration curves were determined from the area values obtained by injecting 10  $\mu$ L of chloroform:methanol (88:12, vol/vol) serially diluted solutions containing 0.25 to 2  $\mu$ g of glycerophosphoethanolamine (PE), 0.5 to 2.5  $\mu$ g of glycerophosphocholine (PC), 0.25 to 2  $\mu$ g of phosphatidylserine (PS), 0.25 to 2  $\mu$ g of glycerophosphoinositol (PI), and 0.5 to 2.5  $\mu$ g of SM. Each solution was prepared and injected in triplicate. The sum of glycerophospholipids (PE, PI, PS, and PC) and SM concentration was regarded as total polar lipid concentration in the milk. Analysis of variance was performed using the general linear model procedure of Statgraphics Plus version 5 (Statistical Graphics Corp.). Differences between the treatment means were compared at the 5% level of significance using Fisher's least significance difference test.

### **Extraction and Analysis of MFGM Proteins**

Milk fat globule membrane proteins were extracted from fat milk with an SDS-containing solution as previously described (Fortunato et al., 2003). Lysis buffer [63 mM Tris-HCl, pH 9.0, and 2% SDS, supplemented with a protease inhibitor cocktail (Complete Mini, EDTA-free; Roche Diagnostics, Meylan, France)] was added to washed fat globules at a concentration of 1 mL per gram of fat, incubated 1 h at  $20^\circ\text{C}$  with periodical vortexing and centrifuged at  $10,000 \times g$  for 10 min. The floating cream layer was removed and lysates were centrifuged again then stored at  $-80^\circ\text{C}$  for further analysis. Protein concentration (Lowry's method) was assessed with the Bio-Rad RC-DC protein assay (Bio-Rad Laboratories, Marnes-la-Coquette, France) according to manufacturer's instructions. Proteins were resolved by 10% SDS-PAGE and stained with Bio-Safe Coomassie stain (Bio-Rad Laboratories). Quantification of lanes was performed using ImageJ software (<http://rsb.info.nih.gov/ij/>). Alternatively, proteins were electrotransferred onto nitrocellulose for immunoblotting. Blots were saturated with 10% nonfat dry milk and probed 2 h at  $20^\circ\text{C}$  in a Tris buffered saline (TBS) 0.3% Tween-20 (Sigma-Aldrich) with antibodies against mouse adipophilin or bovine butyrophilin (1/5,000, both from Progen Biotechnik GmbH, Heidelberg, Germany), washed extensively in TBS 0.3% Tween-20 and incubated with appropriate secondary antibodies (1/1,500; Sigma-Aldrich). Immunocomplexes were revealed by the enhanced chemiluminescence (ECL) system (Dutscher, Brumath, France). For lactadherin immunodetection, 20  $\mu$ g of MFGM proteins were resolved by 10% SDS-PAGE and electrotransferred onto nitrocellulose. To avoid nonspecific binding, blots were immersed for 1 h at  $20^\circ\text{C}$  in a TBS solution containing 1% (wt/vol) nonfat dry milk and 1% (wt/vol) polyvinylpyrrolidone (Sigma-Aldrich). Blots were briefly rinsed in TBS and probed 2 h in TBS 0.1% Tween-20 (Sigma-Aldrich) with antibodies against bovine lactadherin (1/10,000; kindly provided by J. T. Rasmussen, Department of Molecular Biology, Aarhus University, Aarhus, Denmark). Blots were then washed ( $3 \times 5$  min, followed by a  $3 \times 15$ -min wash) in TBS 0.1% Tween-20 and incubated with goat anti-rabbit secondary antibodies coupled to horseradish peroxidase (1/5,000; Interchim, Montluçon, France). Immunocomplexes were revealed by the ECL system. For stomatin analysis, 50- $\mu$ g proteins were resolved on 10% SDS-PAGE and blotted onto nitrocellulose. Blots were saturated for 1 h at room temperature in TBS containing 1% pyrovinylypyrrolidone and 1% nonfat dry milk and then incubated with anti-human GARP-50



antibody (kindly provided by R. Prohaska, Medical University of Vienna, Vienna, Austria) diluted 1:100 in TBS containing 0.1% Tween-20. After washing, blots were incubated with anti-mouse horseradish peroxidase antibodies diluted 1:10,000 in TBS, washed again, and revealed by the ECL system. A lysate from human erythrocytes (kindly provided by M. Vayssier, Maisons-Alfort, France) was used as a positive control for anti-stomatin antibodies. For mucin 1 (MUC-1) analysis, 50  $\mu$ g of proteins were separated on 6% gels and revealed with the Schiff reagent (Sigma-Aldrich) as previously described (Cebo et al., 2010).

### **Two-Dimensional Differential Gel Electrophoresis (Benzyltrimethyl-n-Hexadecylammonium Chloride/SDS-PAGE) and Image Analysis**

Differential gel electrophoresis (DIGE) experiments involve a 3-dye system to reduce gel-to-gel variability, thus improving quantitation of proteins in different samples. Because MFGM contains large hydrophobic proteins, we based our proteomic study on a nonconventional approach, namely benzyltrimethyl-n-hexadecylammonium chloride (**16-BAC**)/SDS-PAGE (Hartinger et al., 1996). This method, founded on differential detergent solubility of membrane proteins, allows the analysis of proteins that could not be visualized by conventional 2-dimensional electrophoresis (2-DE; isoelectric focusing, followed by SDS-PAGE). For the labeling reaction, pH adjustment was performed with 1.5 M Tris (pH 8.5). Fifty micrograms of SDS-extracted MFGM proteins was labeled with 400 pmol of CyDye (GE Healthcare, Orsay, France) for 30 min. Milk fat globule membrane proteins from A/A or O/O genotypes (8 animals, 4 per genotype) were minimally labeled with Cy3 or Cy5. Dye swatches were performed to avoid any artifact due to labeling. In addition, a pooled sample derived from all MFGM extracts used in the study was minimally labeled with Cy2 (internal control). The reaction was stopped by addition of 1  $\mu$ L of 10 mM lysine for 10 min. All steps were performed on ice and in the dark to avoid bleaching of fluorophores. Equal amounts (50  $\mu$ g) of Cy2, Cy3, and Cy5 minimally labeled proteins were mixed and then precipitated 2 h at  $-80^{\circ}\text{C}$  using 50% acetone. Samples were centrifuged at  $15,000 \times g$  for 15 min at  $4^{\circ}\text{C}$  and then solubilized in the 16-BAC buffer [3.75 M urea, 5% (wt/vol) 16-BAC, 5% (vol/vol) glycerol, and 36 mM DTT plus bromophenol blue as a tracking dye] as previously described (Hartinger et al., 1996). The equilibrated strips were placed on the top of 10% SDS-PAGE gels for second-dimension electrophoresis. Gels were scanned with an Ettan DIGE Imager (GE Healthcare) and images were

analyzed using SameSpots software (Nonlinear Dynamics Ltd., Newcastle, UK). Image quality control was assessed by the software, and all gels were aligned before analysis. Automatic analysis of the whole experiment included SameSpots detection, background detection, and normalization. Results were then validated manually to define spots of interest.

### **In-Gel Digestion and Liquid Chromatography-Tandem Mass Spectrometry Analysis**

Bands from 1D-SDS PAGE gels were cut and in-gel digestion was performed with the ProGest system (Digilab Inc., Holliston, MA) according to a standard trypsin protocol. Gel pieces were washed twice by successive separate baths of 10% acetic acid, 40% ethanol, and acetonitrile. They were then washed twice with successive baths of 25 mM  $\text{NH}_4\text{HCO}_3$ , and acetonitrile (ACN). After gel drying, reduction with 10 mM dithiothreitol in 25 mM  $\text{NH}_4\text{HCO}_3$  and alkylation with 55 mM iodoacetamide in 25 mM  $\text{NH}_4\text{HCO}_3$  were performed. Digestion was subsequently achieved for 6 h at  $37^{\circ}\text{C}$  with 125 ng of modified trypsin (Promega, Charbonnières-les-Bains, France) dissolved in 20% methanol and 20 mM  $\text{NH}_4\text{CO}_3$ . Peptides were extracted successively with 2% trifluoroacetic acid (TFA) and 50% ACN and then with ACN. Peptide extracts were dried in a vacuum centrifuge and suspended in 20  $\mu$ L of 0.08% TFA and 2% ACN. High performance liquid chromatography was performed on an Ultimate 3000 LC system (Dionex, Voisins-le-Bretonneux, France). A 4- $\mu$ L sample was loaded at 20  $\mu$ L/min on a C18 Pep-Map 100 precolumn cartridge (5  $\mu$ m; column: 300  $\mu$ m i.d., 5 mm long; Dionex) and desalted with 0.08% TFA and 2% ACN. After 4 min, the precolumn cartridge was connected to the separating C18 PepMap C18 100 column (3  $\mu$ m; column: 75  $\mu$ m i.d., 150 mm long; Dionex). Buffers were 0.1%  $\text{HCOOH}$ -2% ACN (A) and 0.1%  $\text{HCOOH}$ -80% ACN (B). The peptide separation was achieved with a linear gradient from 0 to 36% B for 18 min, at 300 nL/min. Including the regeneration step at 100% B and the equilibration step at 100% A, 1 run took 50 min. Eluted peptides were analyzed online with a LTQ Orbitrap mass spectrometer (Thermo Electron, Courtaboeuf, France) using a nanoelectrospray interface. Ionization (1.3-kV ionization potential) was performed with liquid junction and a capillary probe (10  $\mu$ m i.d.; New Objective). Peptide ions were analyzed using Xcalibur 2.07 with the following data-dependent acquisition steps: (1) full-scan positive mode spectra were measured between ( $m/z$ ) 300 and 1,600 and (2) tandem mass spectrometry (MS/MS) in linear trap ( $q_z = 0.25$ , activation time = 30 ms, and collision energy

= 45%, where  $qz$  is a dimensionless parameter that determines stability of ion trajectories depending upon their  $m/z$  ratio, the size of the ion trap, and the amplitude and frequency of the fundamental radio frequency; centroid mode). Step 2 was repeated for the 4 most abundant ions detected in step 1. Dynamic exclusion time was set to 90 s.

### Peptide Identification and Quantification

A database search was performed with X! Tandem 2008.02.01 software (<http://www.thegpm.org/TANDEM/>). Enzymatic cleavage was declared as a trypsin digestion with 1 possible miscleavage. Cysteine carboxyamidomethylation and methionine oxidation were set to static and possible modifications, respectively. Precursor mass and fragment mass tolerance were 10 ppm and 0.5 Da, respectively. A refinement search was added with similar parameters except that semi-trypsin peptide and possible N-terminal protein acetylation were searched. Few databases were used: the *Capra hircus* database (698 entries, version 8/5/2008 from UniProt, <http://www.uniprot.org/>), the *Mus musculus* database (34,683 entries, version 3/6/2008), the *Bos taurus* database (12,099 entries, version 3/10/2008), and a contaminant database (see Supplementary File, available online at <http://www.journalofdairyscience.org/>). Only peptides with an expected value (**E-value**) smaller than 0.1 were reported.

Identified proteins were filtered according to (1) a minimum of 2 different peptides required with an E-value smaller than 0.05 and (2) a protein E-value smaller than  $10^{-4}$ . In the case of identification with only 2 or 3 MS/MS spectra, similarity between the experimental and the theoretical MS/MS spectra was visually checked. To get an idea of protein abundance, we used label-free quantitation: spectral counting (peptide counting) and peak area calculation with expressed sequence tag (EST) sequences *Ovis aries* (no caseins; <http://www.itb.cnr.it/gosh/>; 353,940 entries) and *Capra hircus* protein database (698 entries). For protein quantitation by integration of peak area, peptides specific to the identified proteins were selected, and only their most abundant charge state was analyzed. Semi-tryptic and methionine containing peptides were eliminated to avoid possible decrease in reproducibility related respectively to incomplete digestion and methionine oxidation. Peak area corresponding to the selected peptides was integrated in the extracted ion current (XIC) within a window of  $m/z \pm 0.01$ .

Peptides found in at least 2 samples of a genotype were kept for statistical analysis. Only proteins repre-

sented by at least 2 kept peptides were submitted to ANOVA. For each of them, the following model was used:  $\log(\text{peptide intensity}) = \mu + P_i + G_j + \varepsilon_{ij}$ , where  $\mu$  is the general mean,  $P_i$  is the effect of the  $i$ th peptide of the protein,  $G_j$  is the effect of the  $j$ th genotype (O/O vs. A/A), and  $\varepsilon_{ij}$  is the residual (Valot et al., 2011).

### RNA Isolation and Quantitative Real-Time PCR Analysis

Goats were slaughtered immediately after milking. All experimental procedures were performed in compliance with the policies of INRA's Animal Care Committee. Mammary tissues were collected in the secretory area containing lobulo-alveolar structures (acini). Samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Total RNA were extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). The RNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Rockford, IL) and RNA quality was assessed with Agilent RNA 6000 LabChips (Agilent Technologies Inc., Santa Clara, CA). The RNA integrity numbers of all samples were at least 8.0. Ribonucleic acid (500 ng) was reverse transcribed by the Superscript III reverse transcriptase using an equimolar mix of oligo-dT and random primers (Applied Biosystems Inc., Foster City, CA). Quantitative real-time PCR was performed in triplicate using 10 ng of cDNA with 300-nM primers in a final reaction volume of 25  $\mu\text{L}$  of 1 $\times$  SYBR Green PCR Master Mix (Applied Biosystems Inc.). The primers used were as follows: for lactadherin: GGA CAT GAC AAT TAT GAT CAG TTG GT (forward) and GGT CAA TCT GCA GCC ACT CA (reverse); for stomatin: ACA GGC GCT GGC AAG AAC (forward) and AAA CTC AGA GCA GGT TGC AAA CT (reverse); for ribosomal protein S24 (reference gene): TTT GCC AGC ACC AAC GTT G (forward) and AAG GAA CGC AAG AAC AGA ATG AA (reverse). The PCR cycling conditions were  $95^{\circ}\text{C}$  for 10 min, linked to 40 cycles of  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 1 min. Real-time PCR data were collected by the Applied Biosystems 7900HT Sequence Detection System and calculations for the expression of the different genes were performed with SDS 2.3 software (Applied Biosystems Inc.) using the standard curve method. Dissociation curves gave  $T_m$  values of  $82.2^{\circ}\text{C}$  and  $76.2^{\circ}\text{C}$  for milk fat globule epidermal growth factor 8 (MFG-E8) and stomatin PCR products, respectively. The relative expression for each gene of interest between mammary gland tissues from goats with extreme genotypes at the  $\alpha_{s1}$ -CN locus was tested for significance by a randomized test imple-

mented in the relative expression software tool (REST 2009 V2.0.13; Qiagen), based on the pairwise fixed real-location randomized test (Pfaffl et al., 2002).

## RESULTS

### **Physicochemical Properties of Goat Milk Fat Globules Are Related to the $\alpha_{s1}$ -CN Genotype**

The parameters calculated from the fat globule size distributions at (mean  $\pm$  SD) early ( $26 \pm 9$  d), mid ( $149 \pm 17$  d), and late ( $250 \pm 9$  d postpartum) lactation are presented in Table 1. Results show that, at midlactation, fat globules in milk from animals with strong genotypes for  $\alpha_{s1}$ -CN (A/A goats) were significantly ( $P < 0.0001$ ) larger ( $3.65 \pm 0.19$   $\mu\text{m}$ ; mean  $\pm$  SD) than fat globules in milk from  $\alpha_{s1}$ -CN-deficient animals (O/O goats;  $3.10 \pm 0.24$   $\mu\text{m}$ ). Figure 1A shows the size distribution of fat globules in the milk from animals with extreme  $\alpha_{s1}$ -CN genotypes (midlactation). The size distributions were monomodal, ranging from 0.8 to 10  $\mu\text{m}$  for A/A goats and from 0.7 to 9  $\mu\text{m}$  for O/O goats. The specific surface area was notably higher in animals secreting smaller fat globules (means  $\pm$  SD:  $2.44 \pm 0.16$   $\text{m}^2/\text{g}$  of fat for O/O goats vs.  $1.97 \pm 0.13$   $\text{m}^2/\text{g}$  of fat for A/A goats at midlactation,  $P < 0.0001$ ). The fat globule size was, therefore, inversely correlated to accessible surface of fat globules in milk. In addition, fat production (g/d) was significantly ( $P < 0.0001$ ) higher at midlactation in animals producing high levels of  $\alpha_{s1}$ -CN (means  $\pm$  SD:  $103.5 \pm 24.0$  vs.  $68.7 \pm 15.4$  g/d for A/A and O/O goats, respectively). Interestingly, the size of fat globules was linearly and positively correlated to the amount of fat in the milk at midlactation [Figure 1B;  $R^2 = 0.75$ ; fat content (g/kg) =  $23.04 \times \text{diameter} (\mu\text{m}) - 38.37$ ]. Although we observed the same tendencies for both the mean diameters and surface area of fat globules from O/O and A/A goats at early- and late-lactation stages, results were not significantly different ( $P > 0.05$ ; Table 1). Also, the fat production was not significantly different between O/O and A/A goats at early and late lactation (Table 1). In addition, the absolute value of  $\zeta$ -potential (the global charge of fat globules, which may reflect the glycoprotein and glycolipid composition of the MFGM in milk as well as the mineral composition of the milk) was significantly ( $P < 0.0001$ ) higher for O/O goats compared with A/A goats at all lactation stages (Table 1). These results demonstrate that fat globule parameters are linked to the milk fat secretion level (and hence, to the  $\alpha_{s1}$ -CN genotype) and reveal that the milk fat globules

undergo significant changes in the absence of  $\alpha_{s1}$ -CN protein in the goat.

### **Glycerophospholipid and Sphingolipid Contents of the MFGM Are Affected by the Genetic Polymorphism at the *CSN1S1* Locus**

The quantification of the MFGM polar lipids (glycerophospholipids and sphingolipids) and the determination of the relative proportion for each polar lipid class (Figure 2) were performed using HPLC combined with an evaporative light-scattering detector as previously described (Lopez et al., 2008). The chromatograms show the well-separated classes of the main 5 MFGM polar lipids: PE, PI, PS, PC, and SM (Figure 2A). Results show that, at midlactation, the amount of polar lipids was significantly ( $P < 0.001$ ) higher in goats with the null genotype for  $\alpha_{s1}$ -CN (O/O goats;  $5.97 \pm 0.11$  mg/g of fat; mean  $\pm$  SD) than in goats with strong genotypes for  $\alpha_{s1}$ -CN (A/A goats;  $3.96 \pm 0.12$  mg/g of fat; mean  $\pm$  SD; Figure 2B). The relative higher proportion of polar lipids in the MFGM from goats with the null genotype (O/O) for  $\alpha_{s1}$ -CN can be connected with the increased specific surface area of the fat globules from O/O goats at midlactation. At late lactation, no significant ( $P > 0.05$ ) differences were evidenced in the polar lipid content of the MFGM from goats with extreme genotypes at the *CSN1S1* locus (data not shown). The relative proportion of each class of polar lipids in both genotypes (A/A vs. O/O genotypes) at midlactation is presented in Figure 2C. Whatever the stage of lactation and the  $\alpha_{s1}$ -CN genotype, the most abundant polar lipids in the goat's MFGM were PE, PC, and SM, which is in agreement with literature data (Rodríguez-Alcalá and Fontecha, 2010). At midlactation, concentrations (mg/g of fat) of all individual polar lipids were significantly ( $P < 0.001$ ) higher in the MFGM from O/O (null genotype for  $\alpha_{s1}$ -CN) goats than in the MFGM from A/A (strong  $\alpha_{s1}$ -CN genotypes; Figure 2C) goats. At late lactation, significant differences were still found for PI ( $P < 0.05$ ), PS ( $P < 0.01$ ), PC ( $P < 0.05$ ), and SM ( $P < 0.001$ ) concentrations in the MFGM from goats with extreme genotype at the  $\alpha_{s1}$ -CN locus. Taken together, these data demonstrated that the MFGM polar lipid composition was quantitatively and qualitatively different in the milk from goats with extreme genotype at the *CSN1S1* locus. Although we observed differences in the MFGM polar lipid composition at late lactation stage, the most significant observations ( $P < 0.001$ ) occurred at the midlactation stage.

**Table 1.** Physicochemical characteristics of milk fat globules from goats with extreme genotype at the  $\alpha_{S1}$ -CN locus<sup>1</sup>

| Item                                         | $\alpha_{S1}$ -CN genotype <sup>2</sup> |                  | Statistics |
|----------------------------------------------|-----------------------------------------|------------------|------------|
|                                              | A/A                                     | O/O              |            |
| Size parameter                               |                                         |                  |            |
| Early lactation                              |                                         |                  |            |
| Dmode <sup>3</sup> ( $\mu\text{m}$ )         | 4.02 $\pm$ 0.25                         | 3.85 $\pm$ 0.23  | NS         |
| Surface area ( $\text{m}^2/\text{g}$ of fat) | 1.71 $\pm$ 0.11                         | 1.82 $\pm$ 0.11  | NS         |
| Midlactation                                 |                                         |                  |            |
| Dmode ( $\mu\text{m}$ )                      | 3.65 $\pm$ 0.19                         | 3.10 $\pm$ 0.24  | ***        |
| Surface area ( $\text{m}^2/\text{g}$ of fat) | 1.97 $\pm$ 0.13                         | 2.44 $\pm$ 0.16  | ***        |
| Late lactation                               |                                         |                  |            |
| Dmode ( $\mu\text{m}$ )                      | 3.0 $\pm$ 0.3                           | 2.9 $\pm$ 0.3    | NS         |
| Surface area ( $\text{m}^2/\text{g}$ of fat) | 2.5 $\pm$ 0.1                           | 2.7 $\pm$ 0.3    | NS         |
| $\zeta$ -Potential (mV)                      |                                         |                  |            |
| Early lactation                              | -11.4 $\pm$ 1.1                         | -13.0 $\pm$ 1.2  | ***        |
| Midlactation                                 | -10.1 $\pm$ 1.0                         | -12.0 $\pm$ 1.1  | ***        |
| Late lactation                               | -9.4 $\pm$ 1.2                          | -10.8 $\pm$ 1.0  | ***        |
| Fat content (g/kg)                           |                                         |                  |            |
| Early lactation                              | 49.7 $\pm$ 4.7                          | 49.2 $\pm$ 5.1   | NS         |
| Midlactation                                 | 49.6 $\pm$ 4.5                          | 31.8 $\pm$ 4.5   | ***        |
| Late lactation                               | 56.5 $\pm$ 8.4                          | 42.7 $\pm$ 9.7   | *          |
| Fat production (g/d)                         |                                         |                  |            |
| Early lactation                              | 96.6 $\pm$ 1.8                          | 107.4 $\pm$ 15.8 | NS         |
| Midlactation                                 | 103.5 $\pm$ 24.0                        | 68.7 $\pm$ 15.4  | ***        |
| Late lactation                               | 64.4 $\pm$ 15.2                         | 48.1 $\pm$ 13.1  | NS         |

<sup>1</sup>The fat globule size and  $\zeta$ -potential values were measured at early (26  $\pm$  9 d; n = 3 per genotype), mid (149  $\pm$  17 d; n = 10 per genotype), and late lactation (250  $\pm$  9 d; n = 6 per genotype). Distributions of fat globule size were determined by laser light scattering using a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The  $\zeta$ -potential was determined by the laser Doppler electrophoresis method using a Zetasizer 3000HS (Malvern Instruments Ltd.).

<sup>2</sup>A/A = goats homozygous for  $\alpha_{S1}$ -CN, allele A; O/O = goats homozygous for  $\alpha_{S1}$ -CN, allele O ( $\alpha_{S1}$ -CN null animals).

<sup>3</sup>Dmode = modal diameter.

\*\*\* $P$  < 0.0001; \* $P$  < 0.05 (results of the ANOVA: probability of  $F$ -test).

### Major MFGM Proteins MUC-1, Butyrophilin, and Adipophilin Are Equally Expressed in the MFGM, Whatever the $\alpha_{S1}$ -CN Genotype

We have recently provided a thorough description of MFGM proteins from goat milk (Cebo et al., 2010). The characteristic pattern of MFGM proteins from A/A and O/O goats in 10% SDS-PAGE is shown in Figure 3A. The protein profile displays several major bands identified by peptide mass fingerprinting (PMF) with matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) as FA synthase, xanthine oxidase, butyrophilin, lactadherin, and adipose differentiation-related protein (adipophilin). Representative patterns of MFGM proteins were comparable in the milk from goats with extreme genotype at the *CSN1S1* locus (compare Figure 3A, lanes 1–3 and lanes 4–6). However, an additional lane of about 30 kDa was systematically found in the MFGM protein extracts from goats with a null genotype at the  $\alpha_{S1}$ -CN locus (Figure 3A, asterisk). Coomassie-stained gels quantification by ImageJ software did not support any significant

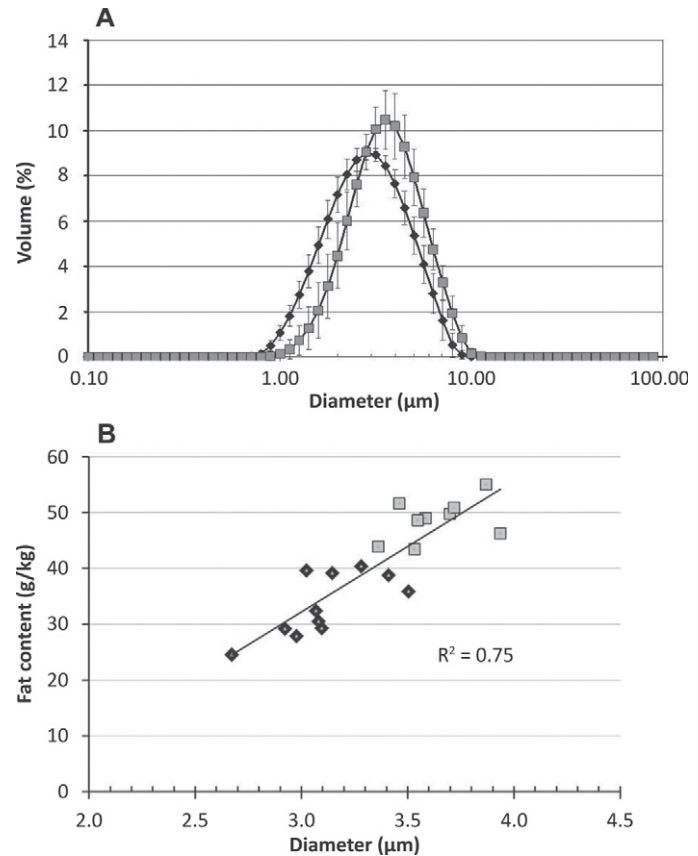
variation for the main MFGM proteins between goats with extreme genotype for  $\alpha_{S1}$ -CN, except for the band attributed to lactadherin, which appeared perceptibly more intense in MFGM extracts from animals deficient for  $\alpha_{S1}$ -CN (data not shown). Expression of the main MFGM proteins butyrophilin and adipophilin were also investigated in MFGM protein extracts from goats with extreme  $\alpha_{S1}$ -CN genotype by Western blotting experiments as previously described (Cebo et al., 2010). Their expression was not significantly different in the milk from A/A or O/O goats at every lactation stage (data not shown). Finally, real-time quantitative PCR on mammary tissues did not show any significant variations in the expression of transcripts for butyrophilin and adipophilin (data not shown). In addition, expression of MUC-1, a high-molecular weight glycoprotein that does not stain well with Coomassie blue due to its high carbohydrate content, was also compared in the milk from goats with extreme genotype at the *CSN1S1* locus by periodic acid Schiff staining (Cebo et al., 2010). Expression of MUC-1 glycoprotein was comparable in milk from goats displaying strong and null



genotypes for  $\alpha_{s1}$ -CN (data not shown). Taken together, the data revealed that the expression of major proteins of the goat MFGM was not significantly affected by the genetic polymorphism at the *CSN1S1* locus, with the exception of lactadherin, for which expression increased in the MFGM from goats with the null genotype for  $\alpha_{s1}$ -CN (see below).

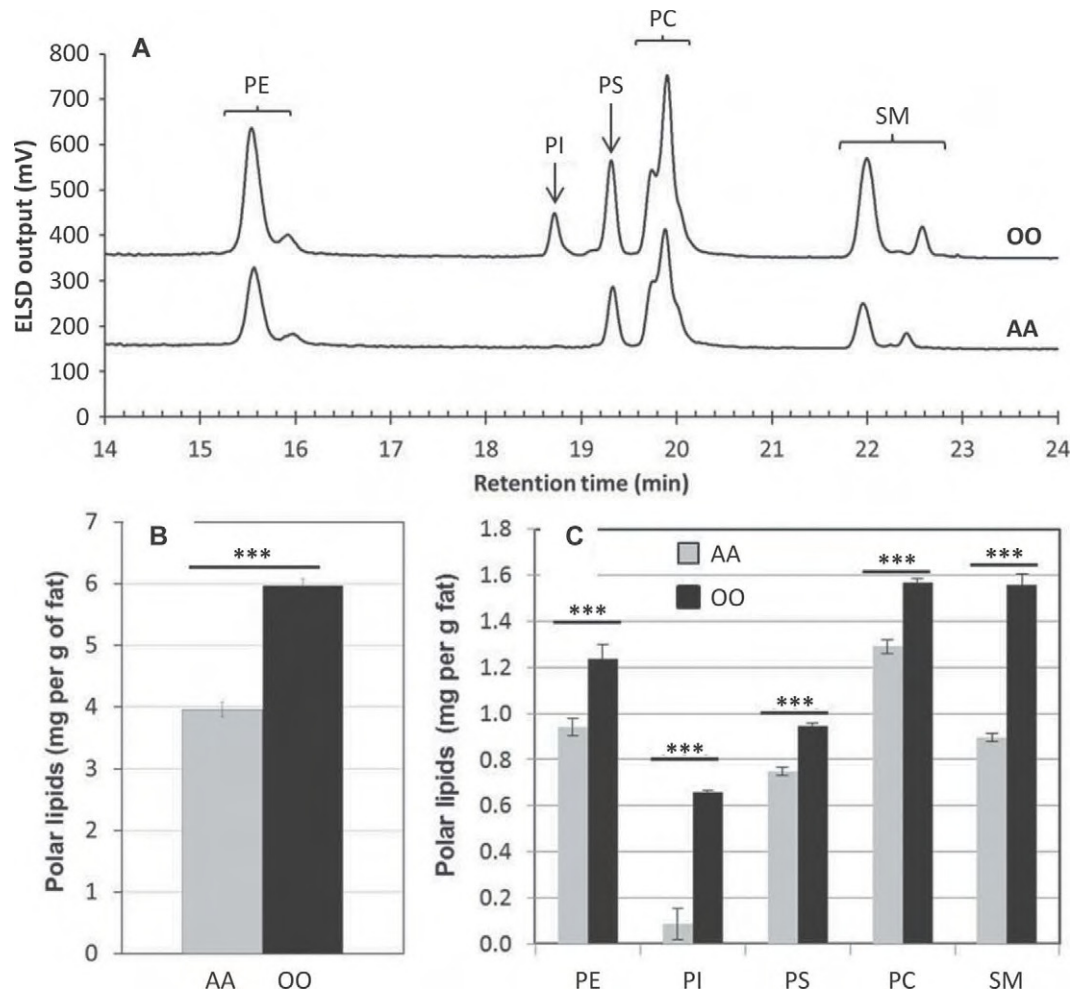
#### Expression of Lactadherin Is Increased in the MFGM from Goats with Null Alleles at the $\alpha_{s1}$ -CN Locus

To get more insights in the MFGM composition in goats with extreme genotypes for  $\alpha_{s1}$ -CN, we performed DIGE-based proteomics on MFGM proteins from A/A and O/O animals. Differential gel electrophoresis experiments involve a 3-dye system to reduce gel-to-gel variability, thus improving quantification of proteins in different samples. Because MFGM contains large hydrophobic proteins, we based our proteomic study on a nonconventional approach, namely 16-BAC/SDS-PAGE (Hartinger et al., 1996). This method, founded on differential detergent solubility of membrane proteins, allows the analysis of proteins that could not be visualized by conventional 2-dimensional electrophoresis (isoelectric focusing, followed by SDS-PAGE). Milk fat globule membrane extracts from 8 animals (4 per genotype) at the early lactation stage were used in the study (see Figure 4A for the 2-dimensional DIGE experimental design). Differential gel electrophoresis experiments results were analyzed using SameSpots software (Nonlinear Dynamics Ltd.) and spots of interest were excised and subjected to LC/MS/MS analysis. The typical pattern of goat MFGM proteins under 16-BAC/SDS-PAGE electrophoresis are presented in Figure 4B. Although resolution of MFGM proteins with this technique is not optimal, it allows visualization of high molecular proteins compared with the conventional 2-dimensional approach. Results of DIGE experiments are depicted in Figure 4B. This study provides evidence for a greater expression of lactadherin in  $\alpha_{s1}$ -CN null animals (spot 2, Figure 4B, montage window), thus confirming our preliminary observations on 1-dimensional SDS-PAGE (Figure 3). Although the O/O versus A/A ratio is weak (1.3), it is statistically significant ( $P = 0.014$ ). To definitely conclude about the expression of lactadherin in animals with extreme  $\alpha_{s1}$ -CN genotypes, we performed Western blot experiments on MFGM extracts using specific antibodies against bovine lactadherin (kindly provided by J. T. Rasmussen, Aarhus University, Aarhus, Denmark). Figure 4C confirmed that, at the early lactation stage, lactadherin protein is slightly increased in the MFGM from goats deficient for the  $\alpha_{s1}$ -CN allele. Also, lactadherin mRNA



**Figure 1.** Fat globule size distribution in the milk from goats with extreme genotype at the  $\alpha_{s1}$ -CN locus. (A) The fat globule size distributions in the milks (evening + morning milking) were determined by laser light scattering using a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Mean values (3 measurements for each sample,  $n = 10$  samples per genotype) are presented for goats homozygous for  $\alpha_{s1}$ -CN, allele A (A/A; gray squares) and goats homozygous for  $\alpha_{s1}$ -CN, allele O ( $\alpha_{s1}$ -CN null animals, O/O goats; black diamonds). The size of fat globules in milk was significantly ( $P < 0.0001$ ) related to  $\alpha_{s1}$ -CN genotype at midlactation ( $149 \pm 17$  d postpartum), with strong genotypes (A/A) producing larger fat globules ( $3.65 \mu\text{m} \pm 0.19$ ) than null genotypes (O/O) for  $\alpha_{s1}$ -CN ( $3.10 \mu\text{m} \pm 0.24$ ). Results are expressed as mean values  $\pm$  SD calculated for milk from individual goats. (B) Linear relationship between the fat content (g/kg) and the modal diameter (Dmode,  $\mu\text{m}$ ) of fat globules in the milk from goats with extreme genotype for  $\alpha_{s1}$ -CN at midlactation. Milk fat globules are from A/A goats (gray squares); O/O goats (black diamonds).

expression assayed by real-time PCR analyses was also slightly higher in mammary gland extracts from goats with null  $\alpha_{s1}$ -CN genotypes (O/O to A/A ratio = 1.262). However, results were not statistically significant between goats with extreme  $\alpha_{s1}$ -CN genotypes ( $P = 0.44$ ), probably because of large differences between individual animals used in the study. Taken together, these results tend to indicate an increased expression of lactadherin in  $\alpha_{s1}$ -CN goats displaying secretion disorders, therefore suggesting a potential role of lactadherin in milk fat globule secretion from the MEC.

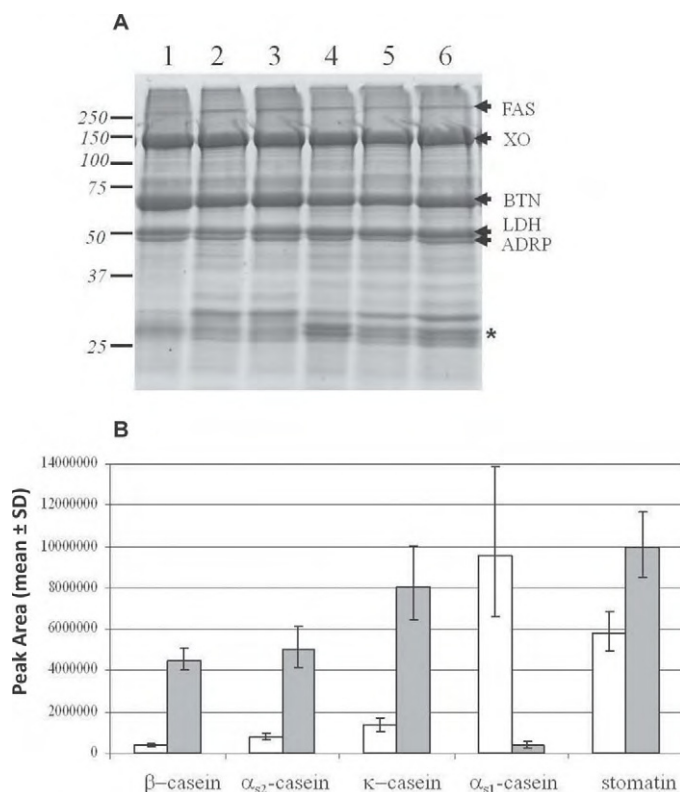


**Figure 2.** Comparison of the polar lipid composition of the milk from goats with extreme genotype at the  $\alpha_{s1}$ -CN (*CSN1S1*) locus at mid-lactation. (A) Representative chromatograms of polar lipids extracted from goats homozygous for  $\alpha_{s1}$ -CN, allele A (A/A goats; strong  $\alpha_{s1}$ -CN genotypes) or goats homozygous for  $\alpha_{s1}$ -CN, allele O (O/O goats; null genotypes for  $\alpha_{s1}$ -CN) milk. ELSD = evaporative light-scattering detector. (B) Total polar lipids (mg/g of fat) in the milk fat globule membrane (MFGM) from A/A or O/O goats. (C) Concentration of individual polar lipids (glycerophospholipids and sphingolipids) in the MFGM from A/A (in gray) or O/O (in black) goats. Results are expressed as mean values  $\pm$  SD calculated for milk from individual goats. PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine; SM = sphingomyelin. Results of ANOVA: \*\*\* $P < 0.001$ .

### **Stomatin, a Minor MFGM-Associated Protein, Is About 2-Fold Overexpressed in $\alpha_{s1}$ -CN-Deficient Goats**

The 30-kDa additional lane in  $\alpha_{s1}$ -CN-deficient animals (see Figure 3A, asterisk) was picked, digested by trypsin, and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The corresponding region in SDS-PAGE of MFGM extracts from animals with strong  $\alpha_{s1}$ -CN genotype was similarly handled (3 animals per genotype, early lactation). Quantification of proteins in both samples was performed by spectral counting (Valot et al., 2011). This label-free MS-based approach, based on total MS/MS spectra counts of peptides from a given protein,

allows the measurement of relative abundance between proteins in complex mixtures. The accuracy and the reproducibility of the method by using 3 sampling statistics have been demonstrated in several studies (Gao et al., 2008; Wang et al., 2008). In addition to the spectral counting approach, which gives an idea of the most important variation of protein expression, peak area data improve the detection accuracy for protein changes (Gao et al., 2008). We, therefore, performed statistical analysis of peak area calculations on MS spectra. Peptides specific to the identified proteins were selected and the peak area corresponding to the selected peptides was integrated within a window of  $m/z \pm 0.01$ . Differentially expressed proteins in MFGM extracts from animals with extreme genotypes for  $\alpha_{s1}$ -



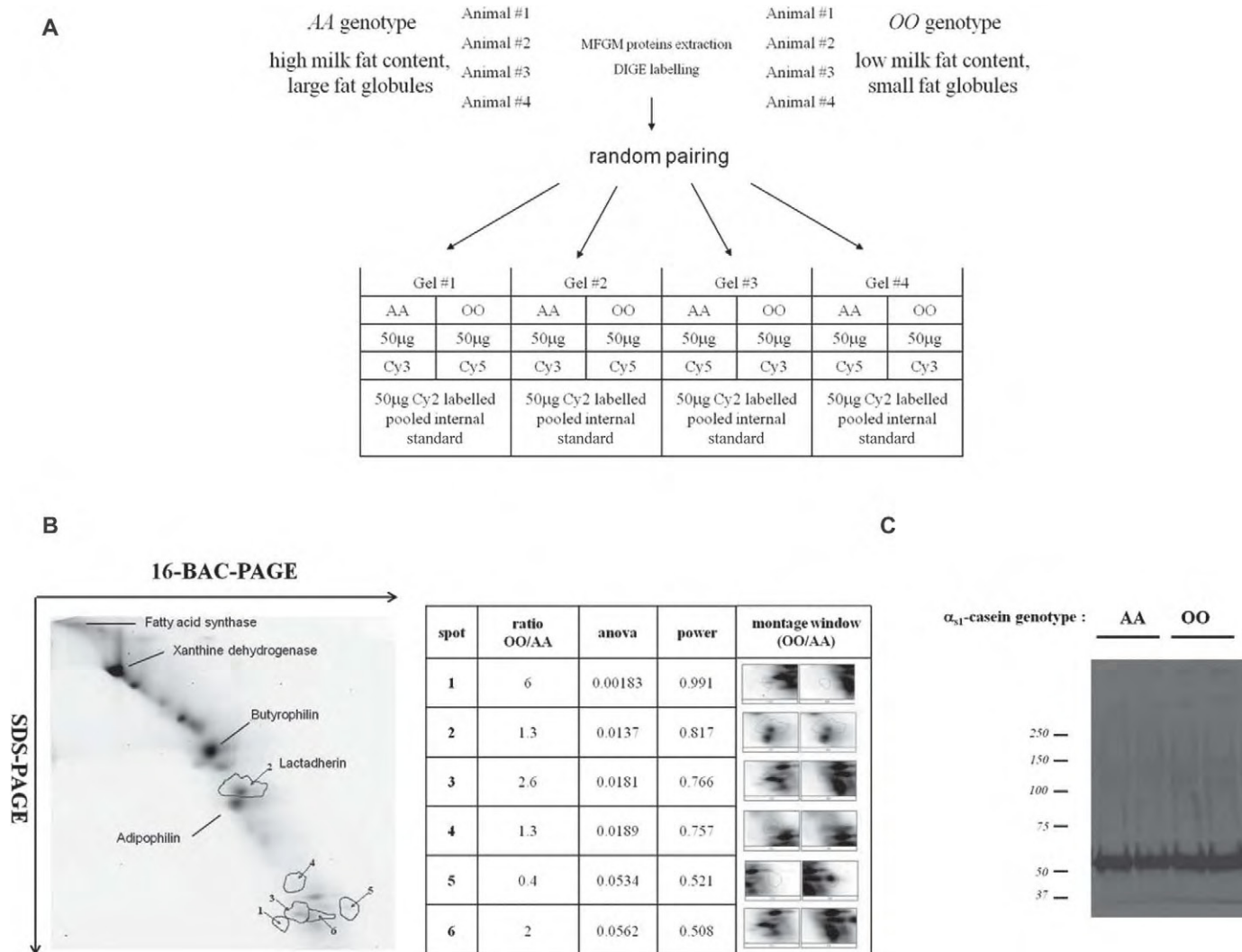
**Figure 3.** Representative pattern of milk fat globule membrane (MFGM) proteins from goats with extreme genotypes at the  $\alpha_{s1}$ -CN locus. (A) Twenty-five micrograms of MFGM proteins (early lactation) was loaded on 10% SDS-PAGE and stained with Bio-Safe Coomassie stain (Bio-Rad Laboratories, Marnes-la-Coquette, France). The MFGM proteins are from (lanes 1–3) goats homozygous for  $\alpha_{s1}$ -CN, allele A (A/A genotypes) and (lanes 4–6) goats homozygous for  $\alpha_{s1}$ -CN, allele O (O/O genotypes). For identification, proteins were in gel digested by 0.2  $\mu$ g of trypsin, and peptide mixtures were subjected to mass spectrometry matrix-assisted laser-desorption ionization time-of-flight (MS MALDI-TOF) analysis. Molecular mass ( $M_r$ ) protein standards (kDa) are indicated to the left of the panel. Note the presence of an additional lane of about 30 kDa in  $\alpha_{s1}$ -CN null genotypes (asterisk, lanes 4–6). (B) Quantitative analysis of proteins of MFGM extracts from goats with extreme genotype at the  $\alpha_{s1}$ -CN locus. The additional 30-kDa lane in animals deficient for  $\alpha_{s1}$ -CN (see Figure 3A, asterisk) was picked, digested by trypsin, and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The corresponding region in SDS-PAGE of extracts from animals producing high levels of  $\alpha_{s1}$ -CN was similarly handled. Peptide peak areas are depicted in white for A/A goats and in gray for O/O goats. Note that, in accordance with the differential gel electrophoresis (DIGE) experiment, most of the proteins associated with smaller fat globules produced by deficient animals were caseins. Quantification of peptides peak area also reveals higher expression of stomatin in  $\alpha_{s1}$ -CN null animals. Results are expressed as mean values  $\pm$  SD calculated for milk from individual goats. FAS = FA synthase; XO = xanthine oxidase; BTN = butyrophilin; LDH = lactadherin; ADRP = adipose differentiation-related protein (adipophilin).

CN in the 30-kDa region are presented in Figure 3B. Results show that, at early lactation, stomatin, a minor protein of the MFGM in bovine and caprine species (Reinhardt and Lippolis, 2006 and our unpublished

data), is about 2-fold expressed in the MFGM from goats with null genotype for the  $\alpha_{s1}$ -CN gene. To conclude about the expression of stomatin in the MFGM from goats with extreme  $\alpha_{s1}$ -CN genotype, Western blot experiments were performed on MFGM extracts using specific antibodies against human stomatin protein (kindly provided by Rainer Prohaska, Medical University of Vienna, Austria). Figure 5 confirms that stomatin protein is overexpressed (about 2-fold) in goats deficient for the  $\alpha_{s1}$ -CN allele. Finally, to confirm our results on MFGM proteins, and because MFGM derives from the MEC (Heid and Keenan, 2005), we initiated real-time quantitative PCR experiments in the mammary gland to evaluate stomatin expression with regard to the genotype for  $\alpha_{s1}$ -CN and, thus, to fat content of milk and size of lipid droplets. The stomatin mRNA sequence is not yet available in the *Capra hircus* taxon. We, therefore, used the bovine stomatin 3'-end mRNA sequence (NM\_001105473) to get expressed sequence tag (EST) sequences in caprine (accession number EV445281) and ovine (accession number EE865394) species. To improve our molecular approach, we aligned bovine, ovine, and caprine sequences using the MultAlin software tool (<http://bioinfo.genotoul.fr/multalin>) to get a conserved region suitable for oligonucleotide design. Quantitative PCR experiments on mammary gland extracts demonstrated that, at midlactation, stomatin mRNA is about 2-fold overexpressed in animals producing milk with reduced fat globule size and lipid content (O/O to A/A ratio = 1.747,  $P = 0.001$ ). Thus, our expression study combining proteomic and transcriptomic analyses reveals that expression of stomatin is significantly increased in the MFGM from goats deficient for the  $\alpha_{s1}$ -CN allele and displaying lipid secretion disorders, thus providing first evidence for a potential role of stomatin in regulating the secretion of milk lipid droplets in the MEC or their release as fat globules in milk, or both.

### Caseins Are More Closely Associated with Milk Fat Globules from $\alpha_{s1}$ -CN Null Goats

Another striking observation deduced from 2-dimensional DIGE experiments on MFGM protein extracts was the identification of caseins, mainly  $\kappa$ - and  $\beta$ -CN, in many spots showing differential expression between A/A (strong  $\alpha_{s1}$ -CN genotypes) and O/O (null  $\alpha_{s1}$ -CN genotypes) goats (Figure 4 and data not shown). This observation was confirmed by our label-free proteomics experiments on MFGM proteins from goats with extreme genotypes for  $\alpha_{s1}$ -CN (Figure 3). According to our 2-dimensional DIGE experiment results, statistically more  $\beta$ -,  $\alpha_{s2}$ -, and  $\kappa$ -CN were associated with fat



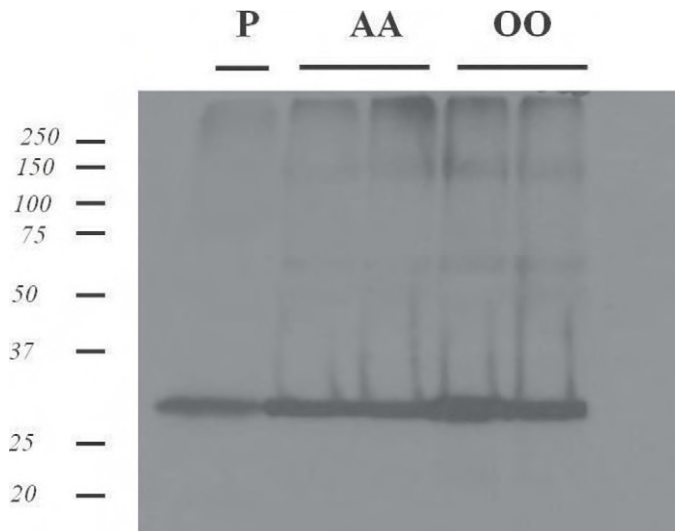
**Figure 4.** Overexpression of lactadherin, a major milk fat globule membrane (MFGM) protein, in the MFGM from goats with null alleles (O/O goats) at the  $\alpha_{s1}$ -CN locus. (A) Two-dimensional differential gel electrophoresis (DIGE) experimental design. Eight animals (4 per genotype) at early lactation were used in this experiment. The MFGM proteins were minimally labeled either with Cy3 or Cy5 dyes (GE Healthcare, Orsay, France) according to manufacturer's instructions. Equivalent aliquots of MFGM extracts used in this study were mixed, therefore constituting the internal standard (Cy2). Note that in gels #3 and #4, labeling of samples was reversed to avoid any bias due to the CyDye coupling reaction. (B) Detail of SameSpots (Nonlinear Dynamics Ltd., Newcastle, UK) analysis. Equal amounts (50 µg) of Cy2, Cy3, and Cy5 minimally labeled MFGM proteins were combined and subjected to 2-dimensional benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC)/SDS-PAGE electrophoresis. Gels were scanned with an Ettan DIGE Imager (GE Healthcare) and data were analyzed using SameSpots software. Differentially expressed spots after analysis are circled. Six spots revealed changes in abundance with ANOVA ( $P \leq 0.05$ ). The figure shows the ratio between O/O and A/A (goats homozygous for  $\alpha_{s1}$ -CN, allele A) genotypes and 2-dimensional fluorescence analysis of the spots revealing an abundance change are depicted (O/O vs. A/A genotypes, montage window). Note the slight but statistically significant ( $P = 0.014$ ) overexpression of lactadherin (spot 2) in  $\alpha_{s1}$ -CN null animals (O/O). (C) Western blot analysis of lactadherin in the MFGM from goats with extreme genotype at the  $\alpha_{s1}$ -CN locus. The MFGM proteins (early lactation) were separated on 10% SDS-PAGE and blotted onto nitrocellulose. After saturation, blots were incubated with anti-PAS 6/7 antibodies and revealed with the enhanced chemiluminescence (ECL) system.

globules from  $\alpha_{s1}$ -CN null animals, although handling of fat globules for MFGM protein extraction was performed in parallel for both genotypes. This suggests that, in null animals, caseins are more closely associated with fat globule membrane or that more caseins are embedded with fat globules during the secretion process in the MEC.

## DISCUSSION

Milk lipids originate from triacylglycerols and cholesterol esters synthesized by enzymes located between the leaves of the ER membrane. They are released as protein-coated cytoplasmic lipid droplets (CLD), which are progressively wrapped up by the apical plas-





**Figure 5.** Overexpression of stomatin, a minor milk fat globule membrane (MFGM)-associated protein, in the MFGM from goats with null alleles (O/O goats) at the  $\alpha_{s1}$ -CN locus. Western blot analysis of stomatin in the MFGM from goats with extreme genotype at the  $\alpha_{s1}$ -CN locus. The MFGM proteins (early lactation) were separated on 10% SDS-PAGE and blotted onto nitrocellulose. After saturation, blots were incubated with anti-human stomatin antibodies and revealed with the enhanced chemiluminescence (ECL) system. A lysate from human erythrocytes was used as a positive control (P) for anti-stomatin antibodies. A/A = goats homozygous for  $\alpha_{s1}$ -CN, allele A.

ma membrane. As a consequence, MFGM composition and mechanisms of fat globule secretion from the MEC are closely related. We anticipated that ER disorders observed in  $\alpha_{s1}$ -CN-deficient animals (Chanat et al., 1999) affect the fine composition of the milk fat globule membrane. We show here that the size, the  $\zeta$ -potential, and the fine composition of the MFGM are actually related to the fat content of goat milk and, hence, to the genotype at the *CSN1S1* locus. Implications of the findings are discussed below.

#### **The Genetic Polymorphism at the $\alpha_{s1}$ -CN Locus: Potential Implications for Techno-Functional Properties and Nutritional Value of Goat Milk**

The highly favorable effect of the allele A on protein, casein, and fat contents in goat milk is indisputable (Grosclaude et al., 1994; Martin et al., 2002). Here, we also observed larger size of fat globules for goats displaying a strong  $\alpha_{s1}$ -CN genotype (A/A homozygous goats) than for goats that are deficient for the  $\alpha_{s1}$ -CN allele (O/O homozygous goats). A positive and linear relationship was found between the size of fat globules [modal diameter ( $D_{mode}$ ),  $\mu\text{m}$ ] and the fat content (g/kg) of milk. Such a relationship between fat globule size and fat content has been recently demonstrated in several studies (Couvreur et al., 2006; Argov et al., 2008;

Menard et al., 2010; Mesilati-Stahy et al., 2011). In the goat species, the larger size of fat globules in goats with strong  $\alpha_{s1}$ -CN alleles may be connected to a higher creaming ability of milk. Indeed, an 11% decrease in milk fat content was followed by a 49% decrease in the cream from goats with alleles deficient for  $\alpha_{s1}$ -CN compared with goats displaying strong  $\alpha_{s1}$ -CN alleles (Delacroix-Buchet et al., 1996; Chilliard et al., 2003). Indeed, large fat globules have the capacity to rapidly move up and separate from the aqueous phase to form a cream layer at the surface of milk.

We also report here that, at midlactation, the specific surface area was significantly higher for fat globules from O/O goats than for A/A goats. Consequently, it may be hypothesized that milk fat lipolysis (hydrolysis of fat globule triacylglycerols into FFA) is higher in the milk from O/O goats because of increased lipolytic enzyme-fat interactions. Accordingly, the goat flavor (which results from a higher content of FFA in cheese) is more pronounced in F/F cheeses than in A/A cheeses, especially in the April to June season, which corresponds grossly to the midlactation period (Delacroix-Buchet et al., 1996). In accordance, milk fat globule sizes (and, therefore, specific surface area of fat globules) in milk from F/F goats are halfway between fat globule sizes from A/A (strong  $\alpha_{s1}$ -CN alleles) and O/O (null  $\alpha_{s1}$ -CN alleles) milk (Neveu et al., 2002).

We also demonstrate that the  $\zeta$ -potential of fat globules was significantly ( $P < 0.0001$ ) higher in absolute value for milk from goats deficient for the  $\alpha_{s1}$ -CN allele than for milk from goats with strong  $\alpha_{s1}$ -CN genotypes. This could be explained by differences in the mineral composition of the aqueous phase (in particular, amounts of mineral salts), but also by differences in the MFGM composition. From this point of view, greater adsorption of  $\beta$ - and  $\kappa$ -CN to milk fat globules from O/O goats may explain differences of  $\zeta$ -potential value reported in this study. A less efficient washing of smaller fat globules from O/O goats may explain the greater adsorption of these caseins to the MFGM. Another possible explanation for  $\zeta$ -potential changes of milk fat globules from A/A and O/O goats may be connected to the increased expression of stomatin in goats with null genotypes at the  $\alpha_{s1}$ -CN locus. Indeed, stomatin has been shown to regulate cation transport through the red cell membrane (Stewart et al., 1993).

The polar lipid content of the MFGM was also significantly different in the MFGM from goats with extreme genotypes at the  $\alpha_{s1}$ -CN locus. At midlactation, polar lipids were found to be significantly in higher amounts in the milk fat from goats with null genotypes for the  $\alpha_{s1}$ -CN gene. This may be due to an increased specific surface area of the fat globules from O/O goats and, thus, higher amounts of membrane material in goats

deficient at the *CSN1S1* locus. Although they account for minute quantities of total lipids, milk polar lipids have been found to be of high nutritional, physiological, and health value for the consumer (Spitsberg, 2005; Dewettinck et al., 2008). At midlactation, the main sphingolipid found in milk (SM) accounted for  $1.56 \pm 0.05$  mg/g of fat in O/O goats compared with  $0.89 \pm 0.02$  mg/g of fat in A/A goats. Because sphingolipids are highly bioactive MFGM components, it may be concluded from the data reported here that the MFGM from goats deficient for the  $\alpha_{s1}$ -CN gene is of higher nutritional value than the MFGM from goats with strong genotypes at the *CSN1S1* locus. However, it must be kept in mind that the greater milk fat content of A/A goats negates the greater polar lipid content in the MFGM from O/O goats, thus resulting in similar SM (roughly, 45 mg/kg of milk) concentrations in whole milk between genotypes. Such an observation has been previously reported for SM concentrations in milk from 2 different bovine breeds (Graves et al., 2007).

The size,  $\zeta$ -potential, and MFGM polar lipid composition in the MFGM from goats with distinct genotypes at the  $\alpha_{s1}$ -CN locus were significantly different at midlactation, whereas early- and late-lactation stages did not reveal any statistically significant difference with regard to these milk fat globule parameters. We noticed that the individual variability of goats was more important at the lactation onset and drying off, thus probably precluding substantial differences to be found between goats. However, at peak lactation (roughly, 5 mo after parturition), these differences must be kept in mind when attempting to exploit techno-functional or nutritive value of milk from dairy goats for which no precise information on the  $\alpha_{s1}$ -CN genotype is available.

### **Genetic Polymorphism at the *CSN1S1* Locus in the Goat as a Powerful Tool to Unravel Mechanisms of Lipid Secretion in the MEC**

Fat in milk is secreted as a lipid core surrounded by a complex membrane (MFGM) arising from the MEC itself (Mather and Keenan, 1998). As a consequence, MFGM composition and mechanisms of fat globule secretion from the MEC are closely dependent. Because of the extensive genetic polymorphism described at the  $\alpha_{s1}$ -CN locus and linked to secretory pathway disorders, the goat model, therefore, represents a powerful tool to unravel mechanisms of lipid droplet secretion in the MEC (Chanat et al., 1999). Indeed, milk lipids originate from triacylglycerols and cholesterol esters synthesized by enzymes located inside the ER membrane. They are released as protein-coated CLD, which are progressively enveloped by the apical plasma membrane. Although lipid and protein synthesis take

place at distinct sites of the ER, numerous studies have shown that the ER is a single, continuous membrane system comprising the nuclear envelope, peripheral ER sheets studded with polyribosomes (the rough ER), and tubule-like structures constituting the smooth ER. Moreover, inter-connected ER compartments establish a highly dynamic network, especially in polarized cells as mammary cells (Voeltz et al., 2002; Park and Blackstone, 2010; Lavoie et al., 2011). We, therefore, anticipated that ER disorders observed in  $\alpha_{s1}$ -CN-deficient animals affect the fine composition of the MFGM.

The current model for fat globule secretion lies only on 3 MFGM proteins, namely adipophilin, butyrophilin, and xanthine oxidase (McManaman et al., 2007). However, their precise role on lipid droplet secretion are poorly defined and recently challenged by Robenek et al. (2006a). These authors suggested that lipid droplet biosynthesis takes place at specialized cup-shaped regions of the ER, and that adipophilin appears to function in transferring lipids from the ER to the lipid droplet surface (Robenek et al., 2006b). The same authors proposed that the final budding of CLD from the MEC and their release as fat globules in milk is controlled by butyrophilin homotypic interactions rather than xanthine oxidase-butyrophilin-adipophilin interactions as previously hypothesized (Mather and Keenan, 1998; Robenek et al., 2006a, 2009). Thus, precise factors regulating the formation and secretion of CLD from the ER still remain to be elucidated.

Here, we show that lactadherin and stomatin, 2 MFGM-associated proteins (Hvarregaard et al., 1996; Reinhardt and Lippolis, 2006), are overexpressed in  $\alpha_{s1}$ -CN null animals displaying secretory pathway disorders. Lactadherin, a major MFGM protein, has been shown to interact with phosphatidyl-L-serine-rich membranes, thus suggesting a potential role for this protein in apoptotic processes (Shi et al., 2004, 2006). Implication of lactadherin in numerous cell-surface associated events has led several authors to suggest a role for this protein in vesicular secretion (Oshima et al., 2002). Membrane-derived vesicles containing lactadherin were found to be high-molecular-mass complexes, suggesting that either homophilic or heterophilic interactions were involved in membrane secretion (Oshima et al., 2002). Remarkably, stomatin, a 31-kDa protein involved in lipid raft segregation that is upregulated in  $\alpha_{s1}$ -CN null animals producing smaller fat globules (the current study), also presents an oligomeric organization, forming self-associated complexes comprising between 9 and 12 monomers (Snyers et al., 1998; Umlauf et al., 2004). At the plasma membrane, stomatin is concentrated in protrusions associated with actin microfilaments and a role for this protein in storage-associated membrane vesiculation of red blood cells has

recently been suggested (Salzer et al., 2008). Moreover, stomatin has been previously shown to be associated with lipid bodies and its role in lipid organization and vesicle trafficking is emerging (Umlauf et al., 2004). It is, therefore, tempting to speculate that stomatin regulates the size of lipid droplets in the MEC. One possible mechanism of the action of stomatin may be to control the membrane curvature, thereby adapting to milk fat level secretion, as it has been suggested that the size of fat globules (and, hence, milk fat content) and MFGM curvature are inversely correlated (Menard et al., 2010). Accordingly, a relationship between membrane curvature and selective association to membrane has been suggested for stomatin (Salzer et al., 2007). Our study demonstrates that MFGM proteomics can provide a snapshot of MEC biology and that analyzing the fine composition of MFGM may help to decipher lipid secretion pathways in the mammary gland (Cebo, 2012).

## CONCLUSIONS

This study demonstrates that, in goats, the genetic polymorphism at the  $\alpha_{s1}$ -CN locus is related to biophysical properties of milk fat globules, including both size and charge, together with the lipid and protein composition of the MFGM. Due to the well-recognized properties of the MFGM, these observations could have an effect on both techno-functional characteristics and nutritional value of goat milk. Moreover, goats with this well-documented genetic polymorphism at the *CSN1S1* locus represent an *in vivo* model for lipid droplet formation and secretion from the MEC. Therefore, because MFGM composition likely reflects those of ER and plasma membrane of the secretory cell, we are now performing large-scale proteomic studies on the MFGM compartment from goats with extreme genotypes at the *CSN1S1* locus to better understand the pathways for lipid secretion in the MEC.

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