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Synthesis of the suspected *trans*-11,*cis*-13 conjugated linoleic acid isomer in ruminant mammary tissue by FADS3-catalyzed Δ 13-desaturation of vaccenic acid

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ABSTRACT

The octadecadienoic conjugated linoleic acid (CLA) isomer with *trans*-11 and *cis*-13 double bonds (*trans*-11,*cis*-13 CLA) has been described in ruminant milk. For now, this specific CLA is suspected to derive exclusively from ruminal biohydrogenation of dietary α -linolenic acid. However, in rodents, the fatty acid desaturase 3 (*FADS3*) gene was recently shown to code for an enzyme able to catalyze the unexpected Δ 13-desaturation of vaccenic acid, producing a Δ 11,13-CLA with all the structural characteristics of the *trans*-11,*cis*-13 isomer, although no commercial standard exists for complete conclusive identification. Because the *FADS3* gene has already been reported in bovine animals, we hypothesized in the present study that an alternative direct FADS3-catalyzed Δ 13-desaturation of vaccenic acid in mammary tissue may therefore co-exist with α -linolenic acid biohydrogenation to explain the final ruminant milk *trans*-11,*cis*-13 CLA presence. Here, we first confirm that the *FADS3* gene is present in ruminant mammal genomic sequence databases. Second, we demonstrate that the Δ 11,13-CLA found in milk fat and the highly probable *trans*-11,*cis*-13 CLA isomer produced by rodent FADS3 possess exactly the same structural characteristics. Then, we show that bovine mammary MAC-T and BME-UV epithelial cells express both *FADS3* and stearoyl-CoA desaturase 1 (*SCD1*) mRNA and are able to synthesize both the suspected *trans*-11,*cis*-13 CLA and *cis*-9,*trans*-11CLA (rumenic acid) isomers when incubated with vaccenic acid. Finally, the concomitant presence of the suspected *trans*-11,*cis*-13 CLA isomer with *FADS3* mRNA was shown in goat mammary tissue, whereas both were con-

versely very low or even absent in goat liver. Therefore, this study provides several lines of evidence that, by analogy with rumenic acid, *trans*-11,*cis*-13 CLA may originate both from ruminal biohydrogenation and from direct FADS3-catalyzed Δ 13-desaturation of vaccenic acid in mammary tissue.

Key words: vaccenic acid, *trans*-11,*cis*-13 conjugated linoleic acid, fatty acid desaturase 3, stearoyl-CoA desaturase 1

INTRODUCTION

Conjugated linoleic acid isomers consist of a group of octadecadienoic acids (18:2) with double bond pairs differing by position and geometrical configuration (Palmquist et al., 2005). In ruminant milk, rumenic acid (*cis*-9,*trans*-11 CLA) is by far (70–90% of total CLA isomers) the most predominant isomer (Lock and Bauman, 2004). Rumenic acid derives partly from ruminal biohydrogenation of linoleic acid (18:2n-6) and mainly from endogenous host Δ 9-desaturation of vaccenic acid (*trans*-11–18:1 or VA; Grünari et al., 2000), itself produced as an intermediate from linoleic and α -linolenic acid (18:3n-3) ruminal hydrogenation (Harfoot and Hazlewood, 1997; Jenkins et al., 2008).

In studies quantifying milk CLA, the *trans*-7,*cis*-9 (Corl et al., 2002; Piperova et al., 2002; Shingfield et al., 2008a) and the *trans*-11,*cis*-13 (Sehat et al., 1999; Kraft et al., 2003; Luna et al., 2005) are described as the second most prevalent isomers. The *trans*-7,*cis*-9 CLA is thought to be exclusively synthesized by the action of tissue Δ 9-desaturase on *trans*-7–18:1, which is produced in the rumen (Corl et al., 2002). By contrast, the *trans*-11,*cis*-13 CLA is for now suspected to be exclusively of rumen origin (Lock and Bauman, 2004). Ruminal biohydrogenation (Figure 1) can indeed convert dietary α -linolenic acid (*cis*-9,*cis*-12,*cis*-15–18:3, or 18:3n-3) to *cis*-9,*trans*-11,*cis*-15-conjugated triene (Harfoot and Hazlewood, 1997; Jenkins et al., 2008), which

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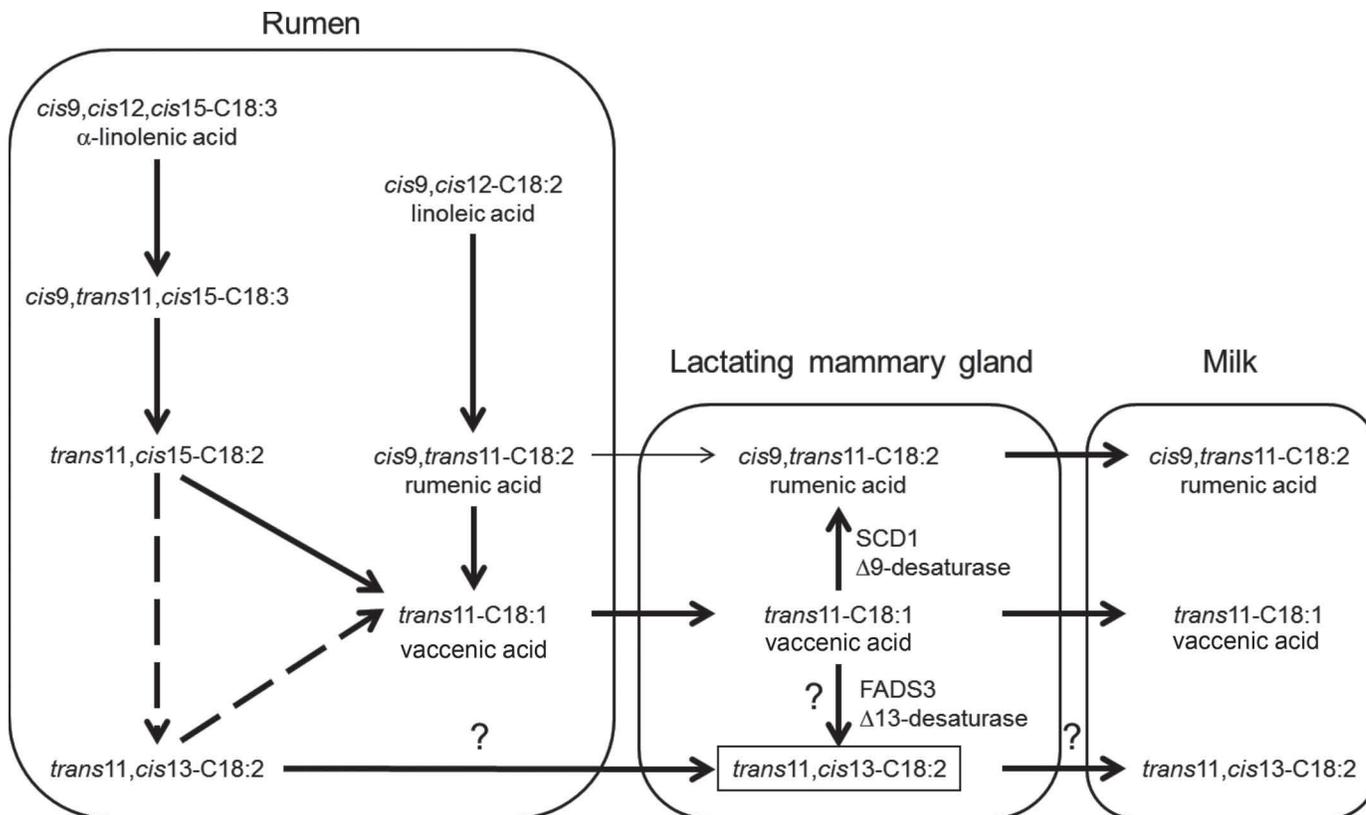


Figure 1. Suspected metabolic pathways leading to the presence of suspected *trans*-11,*cis*-13 CLA in ruminant milk. Ruminal biohydrogenation can convert dietary α -linolenic acid (*cis*-9,*cis*-12,*cis*-15-18:3) to *cis*-9,*trans*-11,*cis*-15-conjugated triene, which is subsequently metabolized to *trans*-11,*cis*-15-18:2 by incomplete bacteria hydrogenation. The step corresponding to the isomerization of *trans*-11,*cis*-15-18:2 to *trans*-11,*cis*-13 CLA has been shown to be catalyzed in vitro by specific *Butyrivibrio fibrisolvens* strains but still remains speculative in vivo. As recently shown in rodents, this fatty acid may also come directly from vaccenate Δ 13-desaturation catalyzed by fatty acid desaturase 3 (FADS3).

is subsequently metabolized to *trans*-11,*cis*-15-18:2 by incomplete bacteria hydrogenation. The last step corresponding to the isomerization of *trans*-11,*cis*-15-18:2 to *trans*-11,*cis*-13 CLA has been shown to be catalyzed in vitro by specific *Butyrivibrio fibrisolvens* strains (Fukuda et al., 2009) but still remains speculative in vivo. In addition to milk, the minor presence of the *trans*-11,*cis*-13 CLA isomer has been described in the digesta (Shingfield et al., 2008b; Toral et al., 2010; Shen et al., 2011) and subcutaneous adipose tissue (Dugan et al., 2007; Petri et al., 2014) of ruminants.

So far, this specific *trans*-11,*cis*-13 CLA isomer has not drawn too much attention, for the following reasons. First, its separation from other CLA isomers present in biological samples (dairy products) remains a considerable analytical challenge (De la Fuente et al., 2006; Christie et al., 2007). Second, no standard of the *trans*-11,*cis*-13 CLA is commercially available (Sehat et al., 1998, 1999), whereas a commercial mixture of *cis*-11,*trans*-13, *cis*-11,*cis*-13, and *trans*-11,*trans*-13 CLA is available (Rioux et al., 2013). Third, great varia-

tions of its amount (0.07–8.00% of total isomers) are reported (Lock and Bauman, 2004). Finally, no function is known for this CLA isomer although it might be physiologically important like other isomers such as *cis*-9,*trans*-11 or *trans*-10,*cis*-12 (Wahle et al., 2004).

Yet, in rodents, the fatty acid desaturase 3 (*FADS3*) gene was recently shown to code for an enzyme able to catalyze the Δ 13-desaturation of VA (Rioux et al., 2013). Structural characterization of the Δ 11,13 CLA produced by this FADS3-catalyzed desaturation strongly suggested that it was the *trans*-11,*cis*-13 CLA isomer. Because the *FADS3* gene has been described in the genomic sequence of the FADS (fatty acid desaturase) gene cluster in cattle (Park et al., 2009; Taniguchi et al., 2014), we hypothesized in the present study that, by analogy with the dual origin of ruminic acid, an alternative and direct host FADS3-catalyzed Δ 13-desaturation of VA (Figure 1) may therefore co-exist with incomplete ruminal biohydrogenation of 18:3n-3 to explain the final presence of the *trans*-11,*cis*-13 CLA in ruminant milk fat.

MATERIALS AND METHODS

Bovine Mammary Epithelial Cell Culture and Incubation with VA

Bovine MAC-T and BME-UV mammary epithelial cells were kindly provided by F. Dessauge (UMR Pegase, INRA Saint-Gilles, France). The BME-UV cells were grown in 5:3:2 (vol/vol/vol) mixture of Dulbecco's modified Eagle medium-F12/RPMI-1640/NCTC35 supplemented with 10% fetal bovine calf serum, 1% lactose, 0.5% lactalbumin hydrolysate, 1.2 mM L-glutathione reduced, 10 μ g/mL L-ascorbic acid, 5 μ g/mL of holo-transferrin, 1 μ g/mL of hydrocortisone and insulin, 0.5 μ g/mL of progesterone, and 1% penicillin/streptomycin. The MAC-T cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine calf serum, 5 μ g/mL insulin, and 1% penicillin/streptomycin.

The ability of the bovine FADS3 protein to Δ 13-desaturate VA was investigated by incubating the cells with VA (Sigma, Saint-Quentin-Fallavier, France) and [1 - 13 C]-VA (chemical purity 95%, isotopic enrichment 99%, synthesized by the Commissariat à l'Énergie Atomique, CEA, Gif-sur-Yvette, France) (Loreau et al., 2000). Incubations with *cis*-11-18:1, *cis*-9-18:1, and *cis*-9,*trans*-11 CLA were also realized. Fatty acid (FA) albuminic complexes were prepared as described (Rioux et al., 2000). The final FA concentration of the incubation medium was 200 μ M (unless indicated otherwise) and albumin concentration was 150 μ M, leading to a FA:albumin molar ratio of 1.3:1. The incubation was initiated by replacing the culture medium with the FA-containing medium. Incubation was then carried out for 24 h at 37°C in a 5% CO₂ atmosphere.

Fatty Acid Analysis

Cellular lipids were extracted with hexane-isopropanol (3:2, vol/vol) after acidification with 1 mL HCl 3 M, as described (Rioux et al., 2015). After saponification for 15 min at 70°C with 1 mL of 0.5 M NaOH in methanol, FA were methylated with BF₃ (14% in methanol) at 70°C for 10 min. The FAME were extracted with pentane and analyzed by GC-MS using an Agilent Technologies 7890N (Bios Analytic, Santa Clara, CA) with a bonded silica capillary column (60 m \times 0.25 mm; BPX 70; SGE). Helium was used as carrier gas (average velocity 24 cm/s). The column temperature program started at 150°C, gradually increased at 2°C/min to 220°C, and held at 220°C for 10 min. Mass spectra were recorded with an Agilent Technologies 5975C inert MSD with triple axis detector.

The mass spectrometer was operated under electron impact ionization conditions (electron energy 70 eV, source temperature 230°C). Data were obtained in scan mode with a mass range of m/z 50–550 atomic mass units (amu). Identification of the FAME was based upon retention times (R_t) obtained for methyl ester of authentic standards, when available. The commercial FA used as standards were ruminic acid (*cis*-9,*trans*-11 CLA), a mixture of *cis*-11,*trans*-13, *cis*-11,*cis*-13, and *trans*-11,*trans*-13 CLA (Matreya, Pleasant Gap, PA), and pure *trans*-11,*trans*-13 CLA (Larodan, Malmö, Sweden). The National Institute of Standards and Technology database (version 2.0) was also sometimes used to identify unknown FA.

Identification of CLA After Their Conversion to 4,4-Dimethyloxazoline Derivatives

To confirm the identity of CLA isomers, FAME were first pre-concentrated by separation on preparative silver ion thin layer chromatography using silicagel H impregnated with 10% (wt/wt) AgNO₃. Separation was performed according to the number and configuration of double bonds, using a mixture of 90/10 (vol/vol) hexane/diethylether for development. At the end of the chromatographic runs, the plates were sprayed with a solution of 10 mg per 100 mL of primuline in acetone (Sigma, Saint-Quentin-Fallavier, France) and visualized under UV light. Bands corresponding to CLA according to a standard of methylated ruminic acid, were scraped off and transferred into a test tube. Collected CLA were then converted to 4,4-dimethyloxazoline (DMOX) derivatives (Guillou et al., 2003; Rioux et al., 2013, 2015) and analyzed by GC-MS in electron impact ionization mode. Briefly, a mild 2-step reaction was used for preparing DMOX. The FAME in dichloromethane (1 mL) were converted to corresponding amides by incubation at 45°C for 30 min with 200 μ L of 2-amino-2-methyl-1-propanol and 100 μ L of potassium *tert*-butoxide in tetrahydrofuran (2:1 vol/vol). The resulting 2-(methylpropanol) amides were isolated by partition between hexane/diethylether (50/50 vol/vol) and water, and then converted to DMOX by treatment with trifluoroacetic anhydride under mild conditions (50°C for 45 min). The column, gas vector, and detector were similar to those used for the analysis of FAME (see above).

Identification of CLA After Their Reaction with 4-Methyl-1,2,4-Triazoline-3,5-Dione

To further confirm the identity of minor CLA isomers, thin layer chromatography pre-concentrated CLA were

also converted to Diels-Alder adducts (Berdeaux et al., 1997; Dobson, 1998) by reaction with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD). The CLA in dichloromethane were mixed with MTAD (1 mg/mL, Santa Cruz Biotechnology, Santa Cruz, CA) at 0°C for 10 s. The reaction was stopped immediately by addition of a 5-fold excess of 1,3-hexadiene (Santa Cruz Biotechnology). The MTAD adducts were analyzed by GC-MS using the same BPX70 column as for FAME analysis but with a temperature gradient starting at 150°C, then gradually increasing at 10°C/min to 260°C, and holding at 260°C for 90 min.

RNA Isolation and Quantitative Reverse-Transcription PCR

Total RNA was extracted from cells with Trizol (Invitrogen). The RNA was converted into cDNA with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The RNA was quantified by Real-Time PCR with the Taqman Universal PCR Master Mix (Applied Biosystem) containing 40 ng of retrotranscribed RNA, 0.5 μ M primers, and 0.25 μ M Taqman probe. Primers and 5'-FAM/TAMRA-3' Taqman probes specific to caprine and bovine desaturase genes are described in Table 1. Amplification was performed by a CFX 96 real-time PCR Detection System (Bio-Rad) over 40 cycles of 95°C for 15 s and 60°C for 1 min (Ramakers et al., 2003). The 18S gene expression was quantified as an endogenous control using the 18S RNA Control kit Yakima-Yellow Eclipse Dark Quencher (Eurogentec, Liège, Belgium) and relative gene expression was determined from the cycle thresholds (Ct) using the $2^{-\Delta Ct}$ method.

Lactating Goat Tissue Samples

Samples of the mammary secretory tissue and liver were collected from 8 multiparous nonpregnant lactating Alpine goats. The experimental design of this study has been previously published (Bernard et al., 2010; Toral et al., 2013). The FA composition of mammary

tissue and liver samples was re-evaluated in the present study using the GC-MS methodology described above, with special focus on the suspected *trans*-11,*cis*-13 CLA. The quantitative reverse-transcription PCR was carried out on mammary tissue and liver mRNA samples using specific primers and probes for goat *FADS1*, *FADS2*, *FADS3*, and stearoyl-CoA desaturase 1 (*SCD1*; Table 1).

Expression of Results and Statistical Analysis

All data values shown represent the mean \pm standard deviation or standard error of the mean for the number of replicates (n) indicated in each figure legend. Student's *t*-test was used to determine the statistical significance between groups, where indicated.

RESULTS AND DISCUSSION

Presence of the *FADS3* Gene Within the *FADS* Cluster in Ruminant Species

The *FADS3* gene was first described in humans, within the genomic structure of the *FADS* gene cluster, which also includes *FADS1* ($\Delta 5$ -desaturase) and *FADS2* ($\Delta 6$ -desaturase) genes and is located in chromosome 11 (Marquardt et al., 2000). Since then, *FADS3* orthologs similarly clustered with *FADS1* and *FADS2* have been identified in rat chromosome 1 (Pedrono et al., 2010), pig chromosome 2 (Taniguchi et al., 2014), baboon chromosome 14 (Park et al., 2009), mouse chromosome 19 (Nakamura and Nara, 2004), and cattle chromosome 29 (Park et al., 2009; Taniguchi et al., 2014). In the present study, genomic database searches in other ruminant species revealed the presence of the *FADS1/FADS2/FADS3* gene cluster with similar order and direction in caprine (chromosome 29, NC_022321.1, *Capra hircus*) and ovine (chromosome 21, NC_019478.1, *Ovis aries*) species, *FADS3* being located tail-to-tail with *FADS2* (Marquardt et al., 2000). In ruminant species, the exon/intron organization of the *FADS3* gene (12 exons) was also similar to that of other mammals,

Table 1. Primers and probes used for quantification of the mRNA levels of fatty acid desaturase 3 (*FADS3*) and stearoyl-CoA desaturase 1 (*SCD1*) genes by real-time reverse-transcription PCR

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	5'-FAM/TAMRA-3' probe
Bovine <i>SCD1</i>	TTGTCCACTTTCTCCTGCTG	GTAGCCATCACTGCCTCTGA	TGGACAGGCAAGCCTCTTCTGTG
Bovine <i>FADS3</i>	GAAAATCTGGCCTACATGCTGG	GGCGTAGAAGCTGGCGG	TGCATGCAGTGGACGGACTTGCTC
Goat <i>FADS1</i>	CCGCCTCCCTCTGCAAG	GGCATTGCCCAAGCT	AGGGTTATGGACCAAAGCAGACGG
Goat <i>FADS2</i>	GCACGGACAAGTGGCTGG	TGCCGGCTGGACCGTT	No probe (Sybr Green)
Goat <i>FADS3</i>	GAAAATCTGGCCTACATGCTGG	GGCGTAGAAGCTGGCGG	TGCATGCAGTGGACGGACTTGCTC
Goat <i>SCD1</i>	TTTCTTCTCTCACGTGGGTTGG	AGCGTAGCACCTTTTCTCTGGA	TGCGCAAACACCCAGCT

although the first 2 exons are lacking in the goat draft genome sequence of the latest assembly (NC_022321.1). Concerning the FADS3 nucleotide sequence and predicted AA sequence (Figure 2), the alignment of goat (XM_013975910.1), sheep (XM_012146315.1), cattle (NM_001083691.2), human (NM_021727.4), and rat (NM_173137.1) sequences exhibited a very high degree of identity (>85%).

Identification and Quantification of the Suspected *Trans-11,Cis-13 CLA* in Cow Milk

We have previously used GC-MS analysis of FAME and DMOX derivatives to accurately discriminate the suspected *trans-11,cis-13 CLA* produced by recombinant rat FADS3 (Rioux et al., 2013) from other CLA isomers and more specifically from the commercially available *cis-11,trans-13*, *cis-11,cis-13*, and *trans-11,trans-13 CLA* isomers. To conclusively identify and quantify the presence of the *trans-11,cis-13 CLA* isomer in ruminant milk, we analyzed the FAME profile from the European certified reference material anhydrous milk fat (BCR164 obtained from Sigma), which corresponds to a pooled sample of cow milks. Figure 3A shows that rumenic acid (*cis-9,trans-11 CLA*), probably co-eluting with *trans-7,cis-9 CLA* and *trans-8,cis-10 CLA* in our experimental conditions (R_t 27.5 min, m/z 294 amu), represented $1.1 \pm 0.2\%$ of total FA in this sample. A well-isolated small peak ($0.06 \pm 0.01\%$ of total FA) with a molecular FAME ion at m/z 294 amu and with the exact same retention time (R_t 28.2 min) as the highly probable *trans-11,cis-13 CLA* produced by recombinant rat FADS3 when incubated with VA (Rioux et al., 2013), was also detected in this sample (Figure 3A).

This peak was also compared with commercially available standards of Δ 11,13 CLA (*cis,trans*, *cis,cis*, and *trans,trans* but not the *trans,cis*; Figure 3A). By comparing the GC retention time of the desaturated product of VA by FADS3 with cow milk fat and these standard (Figure 3A), we first excluded the *cis,cis* (+0.6 min) and *trans,trans* (+0.9 min) isomers with retention times significantly higher than the unknown Δ 11,13 CLA, whereas the *cis,trans* isomer had a slightly lower retention time (−0.2 min). This result suggested that the *cis,trans* isomer could also be discarded. Therefore, the GC retention times offer several arguments to reasonably conclude that this FA is the *trans-11,cis-13 CLA* isomer.

In addition, after conversion to DMOX derivatives, the mass spectrum of this CLA present in cow milk fat (Figure 3B) showed a pattern completely similar to the suspected *trans-11,cis-13 CLA* already characterized

in cells expressing rat FADS3 and incubated with VA (Rioux et al., 2013). Indeed, in addition to the DMOX characteristic intense ions at m/z 113 and 126 amu and molecular ion at m/z 333 amu, a gap of 12 amu was detected between ions containing 10 and 11 carbons (224 and 236 amu) and between ions containing 12 and 13 carbons (250 and 262 amu), showing that this FA is indeed a Δ 11,13 CLA (Figure 3B). More specifically, the ion at m/z 250 amu of this DMOX derivative was significantly less abundant than the ion at m/z 262 amu (Figure 3B), which has already been used to specifically differentiate the suspected *trans-11,cis-13 CLA* from the commercial *cis-11,trans-13 CLA* isomer (Figure 3C) with the nearest R_t of 28.0 min (Figure 3A; Sehat et al., 1998, 1999; Rioux et al., 2013). Therefore, these additional results strongly suggest that this milk FA is the *trans-11,cis-13 CLA*, representing 0.06% of total FA in this sample.

Expression of FADS3 mRNA and Synthesis of Suspected *Trans-11,Cis-13 CLA*

To investigate the potential synthesis of the suspected *trans-11,cis-13 CLA* by the FADS3-catalyzed Δ 13-desaturation of VA in ruminant mammary tissue, we first quantified the relative mRNA expression of *FADS3* and *SCD1* in bovine mammary MAC-T and BME-UV epithelial cells. Figures 4A and 4B show that *FADS3* and *SCD1* mRNA were expressed in these 2 bovine mammary cell models, *FADS3* mRNA being surprisingly 10 times more abundant than *SCD1* mRNA. When incubated with 200 μ M VA, *FADS3* mRNA levels were not modified, whereas *SCD1* mRNA levels decreased, although not significantly (Figures 4A and 4B). This observation is consistent with the described *SCD1* gene expression modulation through the associate gene regulatory protein SREBP-1 downregulation (Keating et al., 2006; Minville-Walz et al., 2012; Jacobs et al., 2013).

The MAC-T and BME-UV mammary epithelial cells were then incubated for 24 h with media supplemented with VA or [$1-^{13}\text{C}$]-VA, and a control medium without FA. Rumenic acid produced by Δ 9-desaturation of VA was easily detected ($1.88 \pm 0.44\%$ of FA in BME-UV cells and $2.22 \pm 1.05\%$ of FA in MAC-T cells, $n = 3$) after incubation with VA (Figures 4B and 4D). A smaller peak ($0.49 \pm 0.24\%$ of FA in BME-UV cells and $0.82 \pm 0.25\%$ of FA in MAC-T cells, $n = 3$) with R_t 28.2 min and FAME molecular ion at m/z 294 amu (incubation with ^{12}C -VA) or m/z 295 amu (incubation with ^{13}C -VA), similar to the suspected *trans-11,cis-13 CLA* previously characterized in milk fat sample (Figure 3A), was also specifically detected (Figures 4C

		1				50
Capra	MGGVGEpd..	...WEPAQR	GAPLPTLRWE	QVRRHNLPD	KWLVIERRVY	
Ovis	MGGVGEpd..	...WEPAQR	GAPLPTLRWE	QVRRHNLPD	KWLVIERRVY	
Bos	MGGVGEpd..	...WEPGQR	GAPLPTLRWE	QVRRHNLPD	KWLVIERRVY	
Homo	MGGVGEpG..	..PREGPAQP	GAPLPTFCWE	QIRAHDPGD	KWLVIERRVY	
Rattus	MGGVGEpGGG	PGPREGPAPL	GAPLPIFRWE	QIRQHDLPD	KWLVIERRVY	
Consensus	MGGVGEpG..	..pregPaq.	GAPLptfrWE	Q!R.H#1PGD	KWLVIERRVY	
		51				100
Capra	DISRWAQRHP	GGSRLLIGHHG	AEDATDAFHA	FHQDLSFVRK	FLQPLLIGEL	
Ovis	DISRWAQRHP	GGSRLLIGHHG	AEDATDAFHA	FHQDLSFVRK	FLQPLLIGEL	
Bos	DISRWAQRHP	GGSRLLIGHHG	AEDATDAFHA	FHQDLSFVRK	FLQPLLIGEL	
Homo	DISRWAQRHP	GGSRLLIGHHG	AEDATDAFRA	FHQDLNFRK	FLQPLLIGEL	
Rattus	DISRWAQRHP	GGSRLLIGHHS	AEDATDAFHA	FHQDLHFVRK	FLKPLLIGEL	
Consensus	DISRWAQRHP	GGSRLLIGHGg	AEDATDAFhA	FHQDL.FVRK	FLqPLLIGEL	
		101				150
Capra	APEEPSQDGP	QNTQLIEDFR	ALRQAVEDMK	LFEAKPAFFG	LLLGHILAME	
Ovis	APEEPSQDGP	QNTQLIEDFR	ALRQAVEDMK	LFEAKPAFFG	LLLGHILAME	
Bos	APEEPSQDGP	QNTQLIEDFR	ALRQAVEDMK	LFEAKPAFFG	LLLGHILAME	
Homo	APEEPSQDGP	LNQQLVEDFR	ALHQAAEDMK	LFDASTFFA	FLLGHILAME	
Rattus	APEEPSQDGA	QNAQLIEDFR	ALRQAAEDMK	LFEADTTFFA	LLLGHILAME	
Consensus	APEEPSQDGP	qNaQL!EDFR	ALrQAaEDMK	LF#A.ptFFa	LLLGHILAME	
		151				200
Capra	VLAWLMIYLL	GPGWLPSTLA	ALILAVSQAQ	SWCLQHDLDG	TSIFRNSRW	
Ovis	VLAWLMIYLL	GPGWLPSTLA	ALILAVSQAQ	SWCLQHDLDG	TSIFRNSRW	
Bos	VLAWLMIYML	GPGWVPSTLA	ALILAISQAQ	SWCLQHDLDG	TSIFRNSRW	
Homo	VLAWLLIYLL	GPGWVPSALA	AFILAISQAQ	SWCLQHDLDG	ASIFKKSWN	
Rattus	LLAWLLVYLL	GPGWVSSVLA	ALILAISQAQ	SWCLQHDLDG	ASIFPKSRW	
Consensus	vLAWL\$!Y\$L	GPGWvpS.LA	AlILA!SQAQ	swCLQHDLDG	aSIF.kSrW	
		201				250
Capra	HLAQQFVMGQ	LKGFSAHWWN	FRHFQHHAKP	NIFHKDPDVT	VAPVFLGEG	
Ovis	HLAQQFVMGQ	LKGFSAHWWN	FRHFQHHAKP	NIFHKDPDVT	VAPVFLGEG	
Bos	HLAQQFVMGQ	LKGFSAHWWN	FRHFQHHAKP	NIFHKDPDVT	VAPVFLGEG	
Homo	HVAQKFVMGQ	LKGFSAHWWN	FRHFQHHAKP	NIFHKDPDVT	VAPVFLGEG	
Rattus	HVAQKFVMGQ	LKGFSAHWWN	FRHFQHHAKP	NIFHKDPDVT	VAPVFLGEG	
Consensus	HvAQqFVMGQ	LKGFSAHWWN	FRHFQHHAKP	NIFHKDPDVT	VAPVFLGEG	
		251				300
Capra	SIEYGKKRR	YLPYNHQHLY	FFLIGPPLLT	LVNFEVENLA	YMLVCMQWTD	
Ovis	SIEYGKKRR	YLPYNHQHLY	FFLIGPPLLT	LVNFEVENLA	YMLVCMQWTD	
Bos	SVEYGKKRR	YLPYNHQHLY	FFLIGPPLLT	LVNFEVENLA	YMLVCMQWMD	
Homo	SVEYGKKRR	YLPYNQOHLY	FFLIGPPLLT	LVNFEVENLA	YMLVCMQWAD	
Rattus	SVEYGKKRR	YLPYNHQHLY	FFLIGPPLLT	LVNFEVENLA	YMLVCMQWTD	
Consensus	S!EYGKKRR	YLPYNhQHLY	FFLIGPPLLT	LVNFEVENLA	YMLVCMQWtD	
		301				350
Capra	LLWAASFYAR	FLLSYVPFYG	IPGALLLFVA	VRVLESHWFV	WITQMNHPIR	
Ovis	LLWAASFYAR	FLLSYVPFYG	IPGALLLFVA	VRVLESHWFV	WITQMNHPIR	
Bos	LLWAASFYAR	FLLSYIPFYG	IPGALLLFVA	VRVLESHWFV	WITQMNHPIR	
Homo	LLWAASFYAR	FFLSYLPFYG	VPGLLFFVA	VRVLESHWFV	WITQMNHPIR	
Rattus	LLWAASFYSR	FFLSYSPFYG	ATGTLFFVA	VRVLESHWFV	WITQMNHIPK	
Consensus	LLWAASFYaR	FfLSY.PFYG	.pG.LLlFVA	VRVLESHWFV	WITQMNHIPK	
		351				400
Capra	EIGHEKHRDW	ASSQLAATCN	VEPSLFIDWF	SGHLNFQIEH	HLFPTMPRHN	
Ovis	EIGHEKHRDW	ASSQLAATCN	VEPSLFIDWF	SGHLNFQIEH	HLFPTMPRHN	
Bos	EIGHEKHRDW	ASSQLAATCN	VEPSLFIDWF	SGHLNFQIEH	HLFPTMPRHN	
Homo	EIGHEKHRDW	VSSQLAATCN	VEPSLFTNWF	SGHLNFQIEH	HLFPRMPRHN	
Rattus	EIGHEKHRDW	ASSQLAATCN	VEPSLFIDWF	SGHLNFQIEH	HLFPTMPRHN	
Consensus	EIGHEKHRDW	aSSQLAATCN	VEPSLFi#WF	SGHLNFQIEH	HLFPTMPRHN	
		401				449
Capra	YRRVAPLVKA	LCAKHGLSYE	VKPFLTALVD	IVRSLKKSNG	VWLEAYLHQ	
Ovis	YRRVAPLVKA	LCAKHGLSYE	VKPFLTALVD	IVRSLKKSNG	VWLEAYLHQ	
Bos	YRRVAPLVKA	LCAKHGLSYE	VKPFLTALVD	IIRSLKKSNG	VWLEAYLHQ	
Homo	YSRVAPLVKS	LCAKHGLSYE	VKPFLTALVD	IVRSLKKSNG	IWLDAYLHQ	
Rattus	YRRVAPLVKA	FCAKHGLHYE	VKPFLTALVD	IIGSLKKSNG	IWLDAYLHQ	
Consensus	YrRVAPLVKa	LCAKHGLsYE	VKPFLTALVD	I!rSLKKSNG#	!WL#AYLHQ	

Figure 2. Alignment of the AA sequences of goat, sheep, cow, rat, and human fatty acid desaturase 3 (FADS3) deduced from their available nucleotide sequences. The alignment of goat (Capra, XM_013975910.1), sheep (Ovis, XM_012146315.1), cattle (Bos, NM_001083691.2), human (Homo, NM_021727.4), and rat (Rattus NM_173137.1) sequences exhibited a very high degree of identity (>85%) (<http://multalin.toulouse.inra.fr/multalin/>).

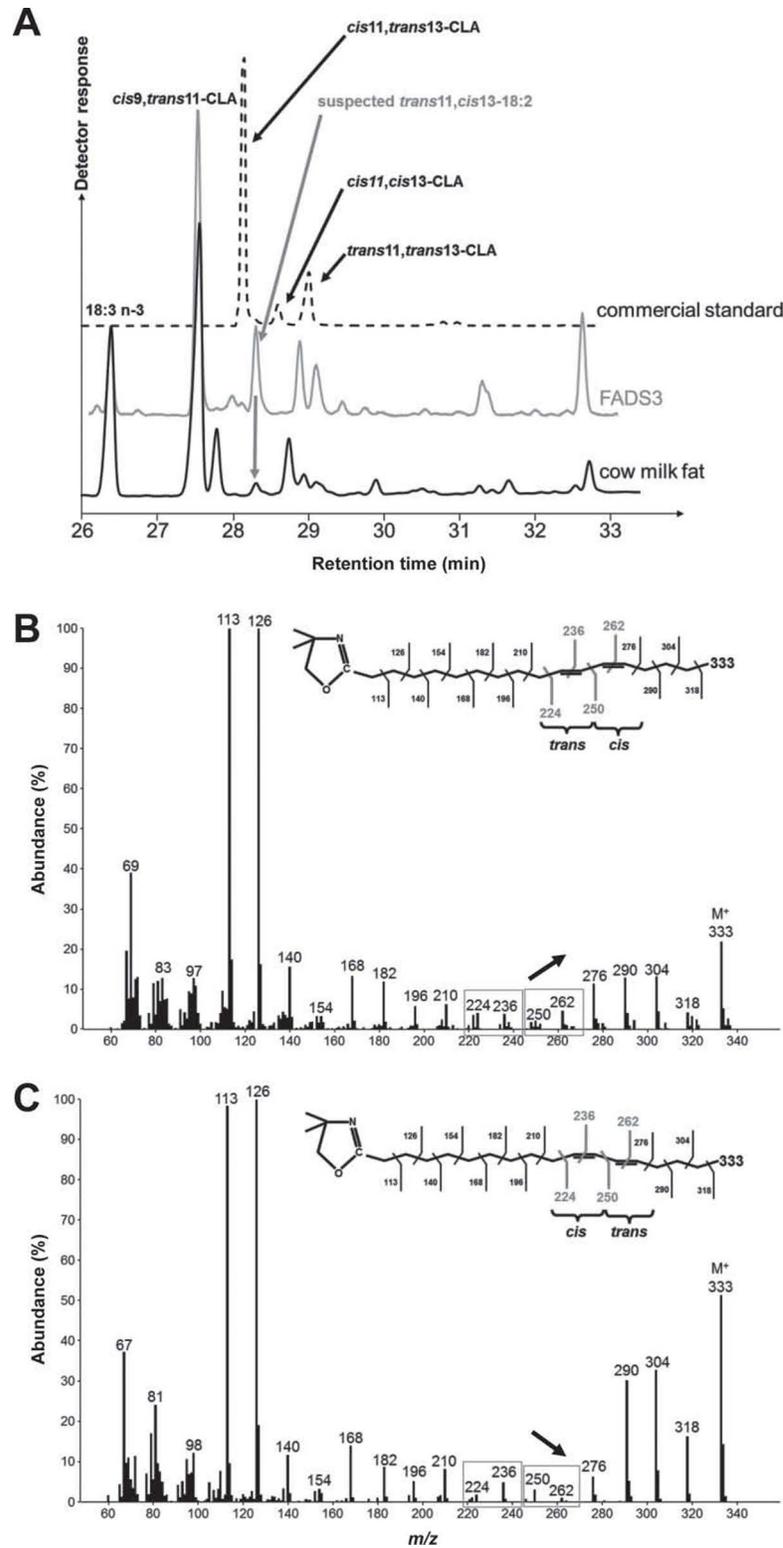


Figure 3. Identification and quantification of the suspected *trans*-11,*cis*-13 CLA in cow milk standard sample. (A) The GC-MS profile of the European certified reference material anhydrous milk fat (BCR164) compared with FAME extracted from COS-7 cells (African green monkey fibroblasts) expressing recombinant rat fatty acid desaturase 3 (FADS3) and incubated with vaccenic acid and a commercial mixture of *cis*-11,*trans*-13; *cis*-11,*cis*-13; and *trans*-11,*trans*-13 CLA as FAME standards. (B) Electron-impact mass spectrum of the 4,4-dimethyloxazoline (DMOX) derivative of the suspected *trans*-11,*cis*-13 CLA from the European certified reference material anhydrous milk fat (BCR164). (C) Electron-impact mass spectrum of the DMOX derivative of the commercial *cis*-11,*trans*-13 CLA.

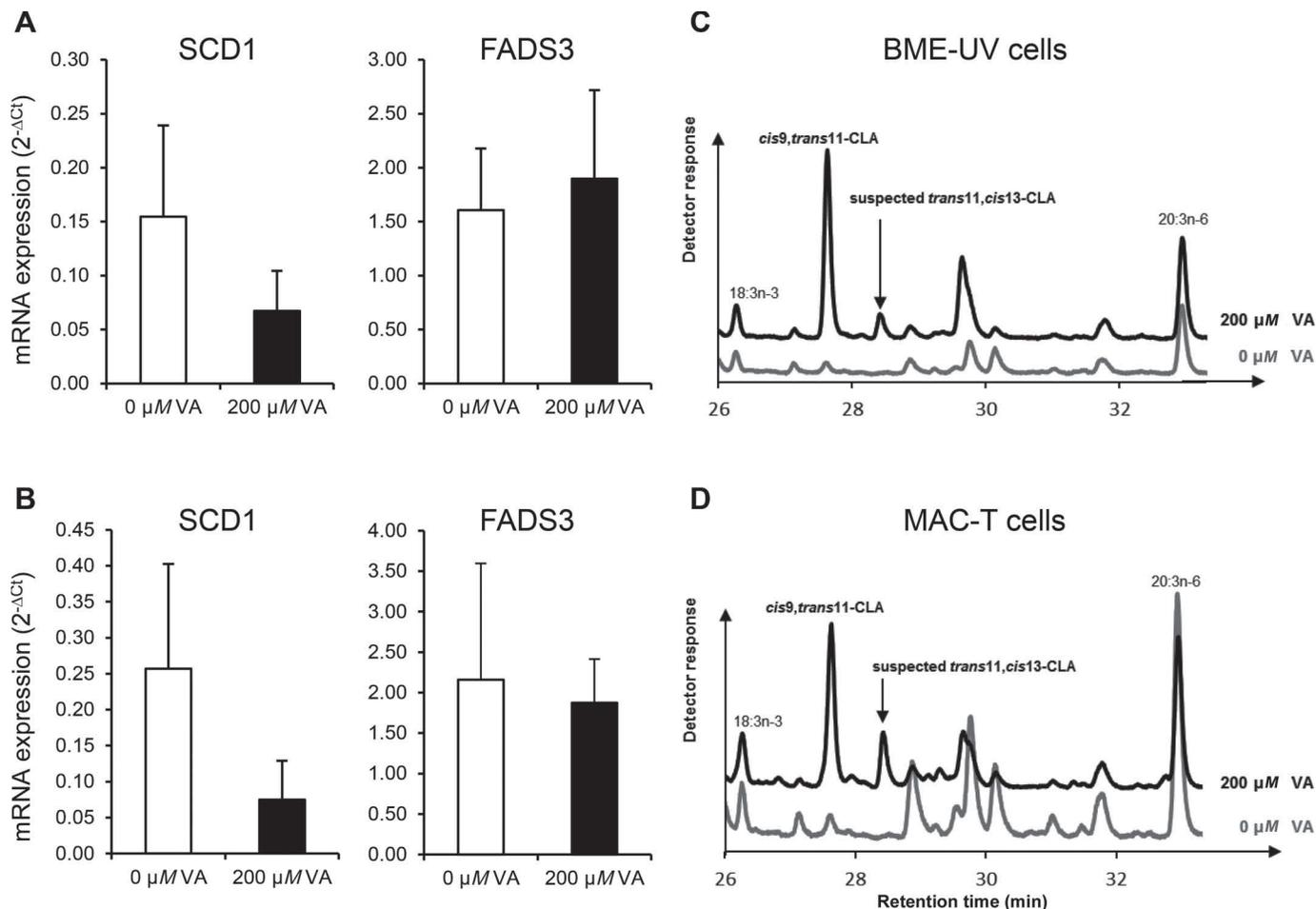


Figure 4. Characterization of bovine BME-UV and MAC-T mammary epithelial cells incubated with vaccenic acid (VA). Cells were incubated for 24 h with albumin-bound VA (200 μ M) or with no fatty acids (FA; 0 μ M VA). The stearyl-CoA desaturase 1 (*SCD1*) and fatty acid desaturase 3 (*FADS3*) mRNA levels were quantified in BME-UV (A) and MAC-T (B) cells. Data are presented as means \pm SEM ($n = 3$). The FAME extracted from bovine BME-UV (C) and MAC-T (D) cells were identified and quantified by GC-MS. The identity of each important FA is indicated above its respective peak. Panels C and D are representative examples of $n = 3$ distinct experiments.

and 4D). Indeed, when incubated mammary epithelial cells were incubated with *cis*-9-18:1, *cis*-11-18:1 or *cis*-9,*trans*-11-18:2, this specific FA was not detected (data not shown).

To clearly identify this CLA isomer obtained from incubation of VA with bovine mammary epithelial cells, it was converted both to MTAD adduct and DMOX derivative (Figure 5), followed by GC-MS analysis. The mass spectrum of the MTAD adduct (Figure 5A) of the suspected *trans*-11,*cis*-13 CLA contained a molecular ion at m/z 407 amu and 3 very characteristic ions at m/z 350, 318, and 222 amu, identifying this CLA as a Δ 11,13 isomer, whereas rumenic acid MTAD adduct (Figure 5B) showed a similar molecular ion at m/z 407 amu but 3 other characteristic ions at m/z 322, 290, and 250 amu (Berdeaux et al., 1997). Figure 5C shows

again that the DMOX mass spectrum of the suspected *trans*-11,*cis*-13 CLA was characterized by 2 gaps between ions containing 10 and 11 carbons (224 and 236 amu) and between ions containing 12 and 13 carbons (250 and 262 amu), locating the double bonds. More specifically, its ion at m/z 250 amu was less abundant than its ion at m/z 262 amu, as already shown in milk fat sample (Figure 3B). Again, these results offer several arguments to reasonably conclude that this FA is the *trans*-11,*cis*-13 CLA isomer.

To conclude, incubation of mammary epithelial cells with VA resulted in a Δ 9-desaturation index [assessed as $RA/(VA+RA+trans-11,cis-13\ CLA)$] of 4.4 and 5.7%, and a Δ 13-desaturation index [assessed as $trans-11,cis-13\ CLA/(VA+RA+trans-11,cis-13\ CLA)$] of 1.1 and 2% in BME-UV and MAC-T cells, respectively.

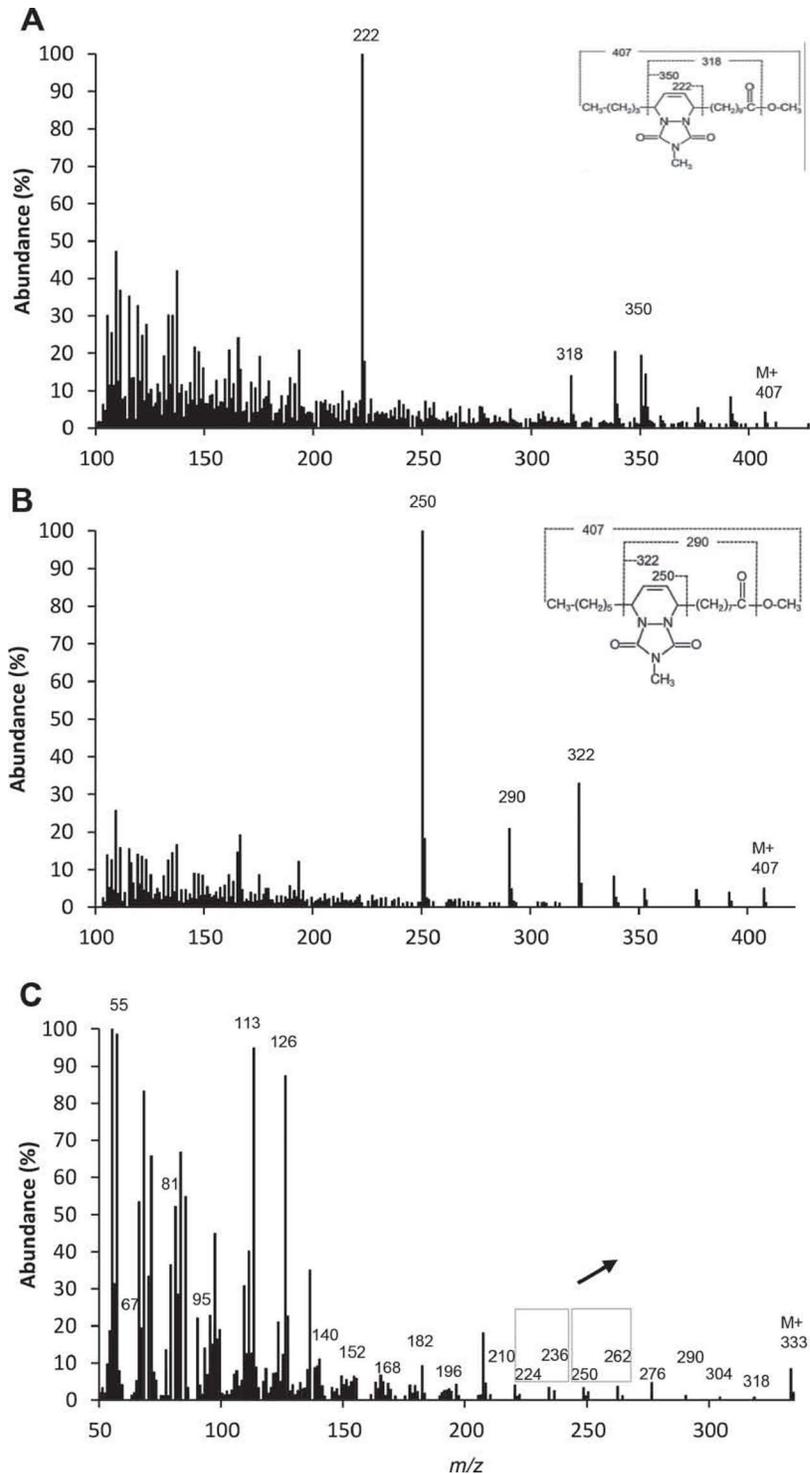


Figure 5. Structural characterization of the CLA obtained after incubation of vaccenic acid (VA) with BME-UV epithelial cells. Electron-impact mass spectrum of the 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) adduct of the suspected *trans*-11,*cis*-13 CLA (A) contains a molecular ion at m/z 407 atomic mass units (amu) and 3 very characteristic ions at m/z 350, 318, and 222 amu, identifying this CLA as a $\Delta^{11,13}$ isomer. Electron-impact mass spectrum of the MTAD adduct of *cis*-9,*trans*-11 CLA (B) also shows a similar molecular ion at m/z 407 amu but 3 other characteristic ions at m/z 322, 290, and 250 amu. The 4,4-dimethylloxazoline mass spectrum of the suspected *trans*-11,*cis*-13 CLA (C) was characterized by 2 gaps (boxed areas) between ions containing 10 and 11 carbons (224 and 236 amu) and between ions containing 12 and 13 carbons (250 and 262 amu), locating the double bonds in position 11-12 and 13-14. Specifically, its ion at m/z 250 amu was less abundant than its ion at m/z 262 amu, as indicated by the arrow, and as shown in the milk fat sample (Figure 3B), which strongly suggests that this fatty acid is the *trans*-11,*cis*-13 CLA isomer.

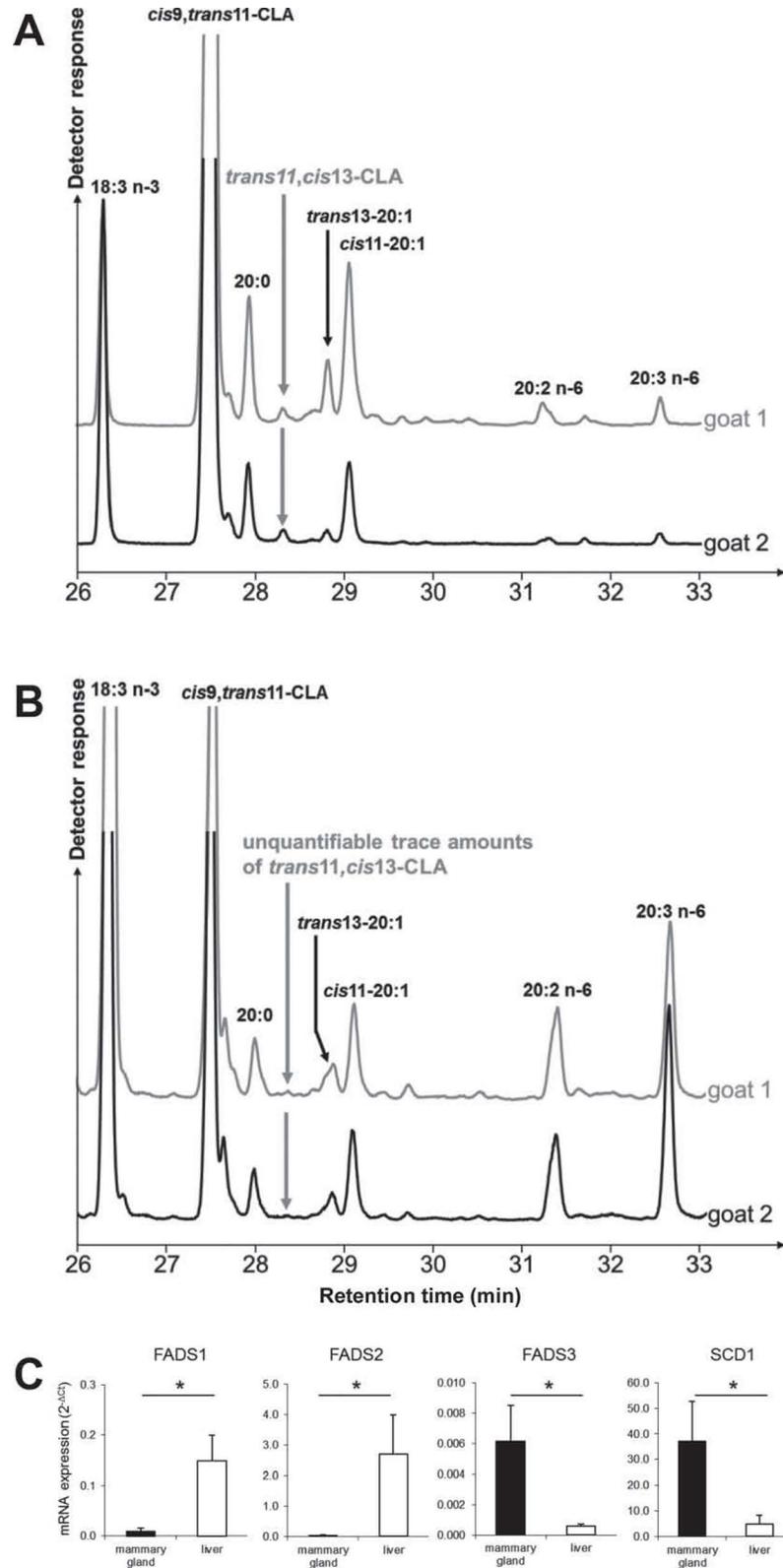


Figure 6. Identification of the suspected *trans*-11,*cis*-13 CLA and quantification of fatty acid desaturase 3 (*FADS3*) mRNA in goat mammary tissue. (A) The GC-MS profile of fatty acids extracted from goat mammary tissue (2 representative examples). (B) GC-MS profile of fatty acids extracted from goat liver (2 representative examples). (C) Comparison of *FADS1*, *FADS2*, *FADS3*, and *SCD1* mRNA levels in goat mammary tissue and liver (n = 8).

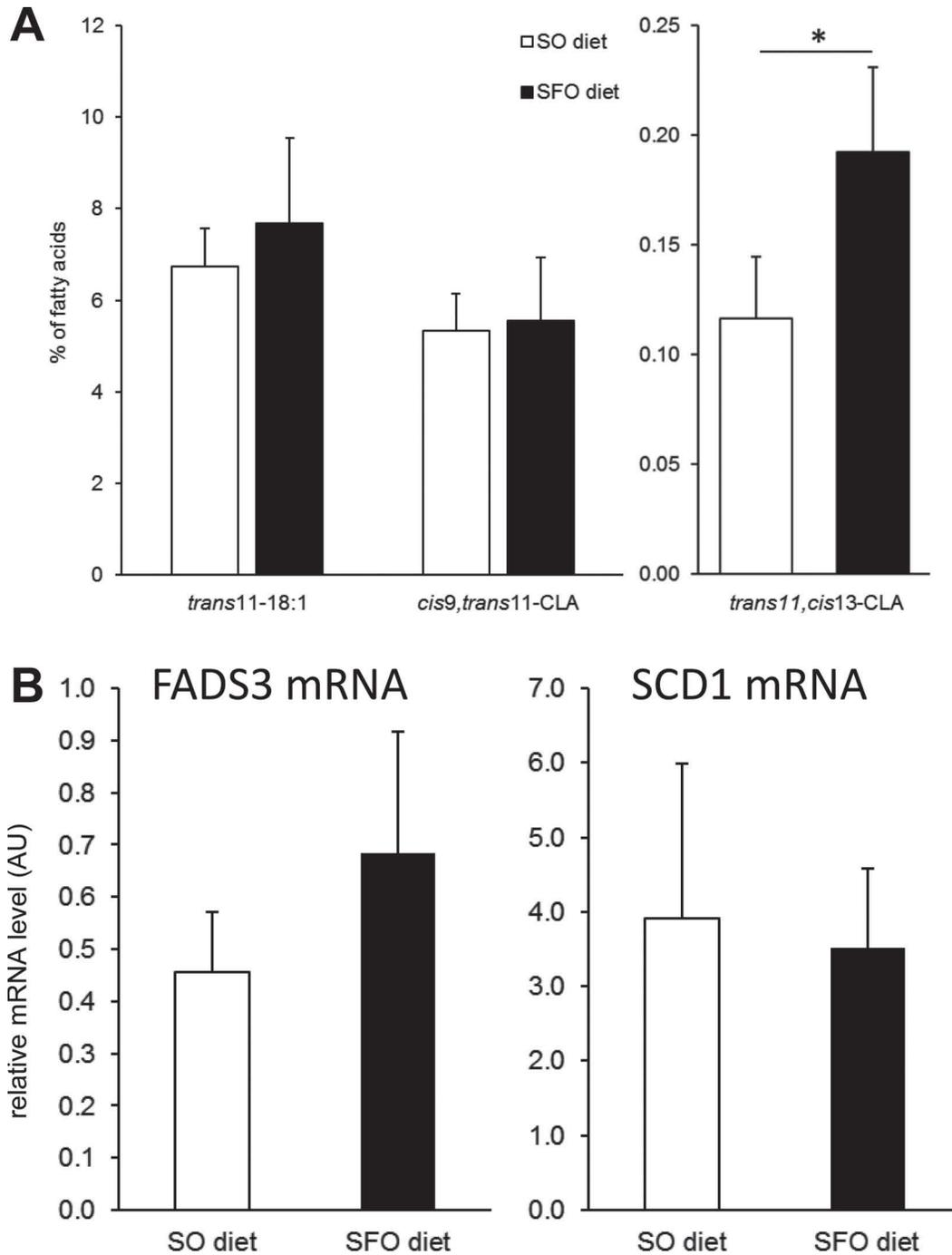


Figure 7. Effect of sunflower-seed oil (SO) and sunflower-seed oil and fish oil (2:1) plus starch from rolled barley (SFO) diets containing exactly the same level of dietary 18:3n-3 on the amount of *trans*-11-18:1, *cis*-9,*trans*-11 CLA and suspected *trans*-11,*cis*-13 CLA and the levels of fatty acid desaturase 3 (*FADS3*) and *SCD1* mRNA in goat mammary tissue. (A) Quantification (% of FA) of *trans*-11-18:1, *cis*-9,*trans*-11 CLA, and suspected *trans*-11,*cis*-13 CLA after GC-MS analysis of FAME extracted from goat mammary tissue. (B) Relative expression of *FADS3* and *SCD1* mRNA levels in goat mammary tissue. Data are presented as means \pm SD ($n = 4$).

FADS3 mRNA and Suspected Trans-11,Cis-13 CLA Concomitant Presence in Goat Mammary Tissue and Absence in Goat Liver

To analyze the potential concomitant presence of *FADS3* mRNA and of the suspected *trans*-11,*cis*-13 CLA in vivo in another ruminant model, we took advantage of tissue samples collected from a study carried out with lactating goats, which has already been published (Bernard et al., 2010; Toral et al., 2013). We re-analyzed the mammary tissue and liver FA compositions, paying particular attention to the suspected *trans*-11,*cis*-13 CLA and additionally quantified *FADS3* mRNA. Figure 6A shows that the suspected *trans*-11,*cis*-13 CLA (R_t 28.2 min) was detected in goat mammary tissue, representing $0.15 \pm 0.05\%$ of total FA ($n = 8$), whereas its trace amounts were not quantifiable in the liver (Figure 6B). Figure 6C additionally shows that *FADS3* and *SCD1* mRNA levels were significantly higher in the mammary secretory tissue than in the liver of goats. Therefore, the higher presence of the suspected *trans*-11,*cis*-13 CLA in goat mammary tissue is consistent with *FADS3* mRNA being expressed in this tissue, compared with very low levels of both suspected *trans*-11,*cis*-13 CLA and *FADS3* mRNA in goat liver. However, in contrast to the results obtained with MAC-T and BME-UV epithelial cells (Figure 4A and 4B), *FADS3* mRNA was about 5,000 times less abundant than *SCD1* mRNA in goat mammary tissue (Figure 6C). This result suggested first that *FADS3* may be less expressed in goats than in cows. Of course, *FADS3* mRNA levels may also be different in mammary tissue samples collected in vivo and in mammary cultured cells. As previously shown (Toral et al., 2013), *FADS1* and *FADS2* mRNA levels were conversely significantly higher in the liver than in the mammary tissue (Figure 6C).

In a second approach to get further information regarding the effect of dietary PUFA on the amount of the suspected *trans*-11,*cis*-13 CLA and *FADS3* mRNA in goat mammary tissue, we took into consideration that the goats were fed during 21 d with 2 experimental diets containing exactly the same amounts of 18:3n-3 but different amounts of other PUFA (Toral et al., 2013). Half of the goats ($n = 4$) were fed 90 g of sunflower oil per day (sunflower oil diet including 27.3 g/kg of DM of 18:2n-6 and 6.2 g/kg of 18:3n-3). Another group of $n = 4$ goats was fed with a combination of 60 g of sunflower oil and 30 g of fish oil (sunflower and fish oil diet including 21.6 g/kg of DM of 18:2n-6, 1.4 g/kg of 20:5n-3, 1.0 g/kg of 22:6n-3 and again 6.2 g/kg of 18:3n-3) plus 59 g/kg of DM of starch from rolled barley. Considering now the effect of the 2 diets, Figure 7A shows that the suspected *trans*-11,*cis*-13 CLA was

significantly higher ($0.19 \pm 0.04\%$ vs. $0.12 \pm 0.03\%$) in the mammary tissue of goats fed the sunflower and fish oil diet compared with goats fed the sunflower oil diet. In addition, a trend toward the increase of *FADS3* mRNA was observed (Figure 7B), even if the difference was not statistically significant ($P = 0.13$) due to the low number of animals. Conversely, no effect of the diets has already been shown on *trans*-11–18:1, *cis*-9,*trans*-11 CLA (Figure 7A), and on *SCD1* mRNA level (Toral et al., 2013), as also re-analyzed in the present study (Figure 7B). Because the 2 diets contained the same amounts of 18:3n-3, we can therefore hypothesize that its ruminal biohydrogenation potentially leading to *trans*-11,*cis*-13 CLA production may have been similar and can speculate that mammary tissue *FADS3* would therefore have been involved in the elevated *trans*-11,*cis*-13 CLA level. Interestingly, the expression of the *FADS3* transcript was also upregulated by dietary long-chain PUFA in baboon liver, whereas other desaturase (*FADS1* and *FADS2*) mRNA levels were downregulated (Reardon et al., 2013). These results are also in agreement with the positive correlation between *trans*-11,*cis*-13 CLA and *trans*-11–18:1 levels described in milk (Lerch et al., 2012).

CONCLUSIONS

A vaccenate Δ 13-desaturase activity has recently been attributed to the *FADS3* enzyme in rodents (Rioux et al., 2013). We hypothesized in the present study that this activity would be common to all mammals, explaining at least partly the presence of the *trans*-11,*cis*-13 CLA in ruminant milk. Indeed, this study provides several lines of evidence that, by analogy with rumenic acid, the *trans*-11,*cis*-13 CLA may originate both from ruminal biohydrogenation and from direct *FADS3*-catalyzed Δ 13-desaturation of VA in mammary tissue.

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