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Characterisation of gene expression related to milk fat synthesis in the mammary tissue of lactating yaks

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This research communication describes the profile of gene expression related to the synthesis of yak milk as determined *via* quantitative reverse transcription polymerase chain reaction (RT-qPCR). Significant up-regulation during lactation were observed in genes related to fatty acid (FA) uptake from blood (*LPL, CD36*), intracellular FA transport (*FABP3*), intracellular FA activation of long- and short-chain FAs (*ACSS1, ACSS2, ACSL1*), *de novo* synthesis (*ACACA*), desaturation (*SCD*), triacyglycerol (TAG) synthesis (*AGPAT6, GPAM, LPIN1*), lipid droplet formation (*PLIN2, BTN1A1, XDH*), ketone body utilisation (*BDH1, OXCT1*), and transcription regulation (*THRSP, PPARGC1A*). In particular, intracellular *de novo* FA synthesis (*ACSS2*, *ACACA,* and *FABP3*) and TAG synthesis (*GPAM*, *AGPAT6*, and *LPIN1*), whose regulation might be orchestrated as part of the gene network under the control of *SERBF1* in the milk fat synthesis process, were more activated compared to levels in dairy cows. However, the genes involved in lipid droplet formation (*PLIN2*, *XDH*, and *BTN1A1*) were expressed at lower levels compared to those in dairy cows, where these genes are mainly controlled by the *PPARG* regulator.

Keywords: Yak, mammary gland, milk fat, lactation, gene expression.

Yaks (*Bos grunniens*) are found extensively on the plateau of Western China in alpine and subalpine regions at altitudes from 2000–5000 m in conditions of extreme harshness (Wiener et al. 2006). For specific regional reasons, yak milk and meat are known to have some unique characteristics compared to those of other ruminants (Wiener et al. 2006; Qiu et al. 2012). One of these characteristics is that yak milk has high fat and protein contents and is produced with low yield. Compared to dairy cow milk, the fat content of yak milk is relatively high at 5–7% fat (g/100 g of milk), while the yield of yak milk is significantly lower at approximately 10% of the yield of dairy cow milk (Wiener et al. 2006). Because of these physical characteristics of yak milk, many researchers have recently attempted to speculate regarding the genetic properties and functional genomics of yak milk synthesis (Qiu et al. 2012; Wang et al. 2016).

Studies of yak milk are important because yak milk is a food for regional Tibetan people; they provide important opportunities for understanding functional genomics for regional yak milk dairies (Wiener et al. 2006). The gene expression profile related to yak mammary milk fat synthesis during the lactation cycle has not previously been investigated. Using a gene expression analysis *via* quantitative reverse transcription PCR (RT-qPCR), we investigated how the expression profiles of milk fat synthesis genes differed in yaks compared to dairy cows. This research will help us understand the influence of gene expression for adaptation to life at high altitude hypoxic environment.

Materials and methods

This study was approved by the Southwest University for Nationalities Institutional Animal Care and Use Committee (permit number: 2011-3-2). Four healthy female yaks from Hongyuan of Sichuan province, China were used. All yaks were fed fresh grass (Hongyuan, DM, 92·7%, CP, 13·4%, and CE, 5·1%) for ad libitum intake during lactation period. Mammary tissue samples (approximately 1 g) were collected by biopsy of the right or left rear quarters at −15, 1, 15, 30, 60, 120 and 240 d relative to parturition (d), as previously described (Bionaz & Loor, 2007). All samples were immediately frozen and stored in liquid nitrogen.

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The milk yield for each yak was recorded during the entire lactation cycle (milking: one time with 400 squeeze at 5 a.m. everyday. recorded on 15, 30, 60, 120, and 180 d).

Part of the yak mammary tissue sample was weighed (100 mg) and immediately homogenised in 1 ml of TRIzol (Invitrogen, Massachusetts, USA). RNA was extracted, and the purity and concentration of RNA were determined by UV/Vis spectrophotometry (Eppendorf, Hamburg, Germany). The 260/280 ratio of the RNA was ≥1·9. The RNA integrity was assessed by 1% gel electrophoresis. All samples clearly presented the 2 expected bands at 18S and 28S, without any evidence of degraded products. The RNA was then diluted to 200 ng/µl and genomic DNA contamination was removed from 600 ng of RNA using a PrimeScriptRT Reagent Kit with gDNA Eraser (TaKaRa Bio, Shiga, Japan). The obtained DNA-free RNA was then diluted with an equal amount before cDNA synthesis. cDNA was synthesised using a PrimeScriptRT Reagent Kit (TaKaRa Bio, Shiga, Japan) following the manufacturer's instructions.

The 40 selected genes related to milk fat synthesis were chosen based on previous studies (McManaman et al. 2007; Bernard et al. 2008; Bionaz & Loor, 2008) (Supplementary Table S1). In this study, the 40 primer sets chosen for RT-qPCR were designed by Bionaz & Loor (2008). The amplicon size (bp) was fixed at 63–151 bp and the melting temperatures ranged between 75 and 87 °C. The sequences of the selected genes were confirmed from the NCBI database (http://www.ncbi.nlm.nih.gov/) and UCSC's Cow (*Bos taurus*) Genome Browser Gateway (http://genome.ucsc.edu/). Information regarding PCR primer sets is summarised in Supplementary Table S1. The amplicon for each primer pair was also purified and sequenced using a 3730 DNA Analyzer (ABI, Vernon, USA) and the results were BLAST searched to verify amplification of the expected gene (Supplementary Table S2). PCR was performed in triplicate for each sample using a CFX96 Real-time System (Bio-Rad, California, USA). A six-point standard curve was generated for each gene by 10-fold dilution of cDNA to determine the efficiency of amplification for each primer pair. The PCR was performed in a 10 µl final volume containing 2 µl of cDNA, 5 µl of SsoFast EvaGreen Supermix (Bio-Rad, California, USA), 0·5 µl each of 10 µM forward and reverse primers, and 2 µl of DNase- and RNase-free water. The instrument was set at 95 °C for 10 min (enzyme activation), followed by 40 cycles at 95 °C for 15 s (denaturation), then the optimal annealing temperature of each primer (56–63 °C, Supplementary Table S1) for 1 min (annealing and extension), 95 °C for 15 s, and 65 to 95 °C for 15 s (melting curve). A negative control without cDNA template was included in each assay.

To calculate the relative quantity (RQ) of each gene, the PCR efficiencies for all genes were calculated using the standard curve according to the following equation (Pfaffl, 2001); Efficiency $(E) = (10^{-1/\text{Slope}})$. The RQ of each gene was calculated using the following equation: $RQ_{\text{Target}} = E^{(\Delta Ct(\text{Target}-15d)-\Delta Ct(\text{Target}))}$, where ΔCt (Target −15 d) is the difference between the Ct value of the target gene at −15 d and the geometric mean of the Ct values of three internal control genes (ICGs) at −15 d and ΔCt (Target) is the difference between the Ct value of the target gene at a single point and the geometric mean of the Ct values of 3 ICGs simultaneously. *MRPS15, RPS23, and UXT* as ICGs for lactating yaks mammary tissue were previously surveyed (Jiang et al. 2016).

The significance was calculated with $\log_2(RQ_{\text{target}})$ and was analysed using the GLIMMIX procedure in SAS (v 9.4, SAS Institute Inc., Cary, USA), with time, normalisation (yes, no), and normalisation with time with yak as a random effect. Differences were evaluated by Tukey's test. The significance (*P*) value with time and the standard error of the mean (SEM) for each gene are listed in Supplementary Table S1.

The RQ of each gene with time and the % mRNA abundance of all investigated milk fat genes in lactating dairy cow mammary tissues were provided by Bionaz & Loor (2008). To analyse the correlation of the gene expression with time between yaks and dairy cows and the % mRNA abundance among genes investigated between yaks and dairy cows, Pearson correlation analysis (Pearson correlation value, ***r***) was conducted using SAS (v 9.4, SAS Institute Inc., Cary, USA).

Milk fat extraction and FA methylation was conducted according to Gomez-Cortes et al. (2009) and were subsequently analysed by gas chromatography (GC-MS system : 6890A-5975C, Agilent, Santa Clara, USA) with an HP88 capillary column (60 m × 250 µm × 0·2 µm). Detailed measurement conditions are described in Supplementary Table S4. The measured FA concentrations were also analysed using the GLIMMIX procedure of SAS (v 9.4, SAS Institute Inc., Cary, USA), with time, normalisation (yes, no), and normalisation with time with yak at random. The data were separated using Tukey's test (Supplementary Table S4).

Results and discussion

The genes and pathways related to milk fat synthesis were determined through pathway analysis. Producing milk fat in ruminants involves many steps, i.e., for long- chain FA (LCFA) synthesis, FAs are taken from the blood and transported inside cells, where the transported FAs are activated, sequentially desaturated and synthesised to TAG. After completion, the lipid molecules form lipid droplets that are exported into milk (Fielding & Frayn, 1998). In another pathway, for short-chain FA (SCFA) synthesis, short-chain carbon sources, such as β-hydroxybutyrate and acetate, are imported inside the cells, where FAs are synthesised using the short-chain carbon sources and are incorporated into TAG through related proteins and then released into milk (Bauman & Davis, 1974, KEGG, http://www.genome.jp).

Yak milk yield and the composition analysis of milk fat

We investigated the daily yield, FA concentration, and composition of yak milk (Fig. 1). The yak milk yield significantly

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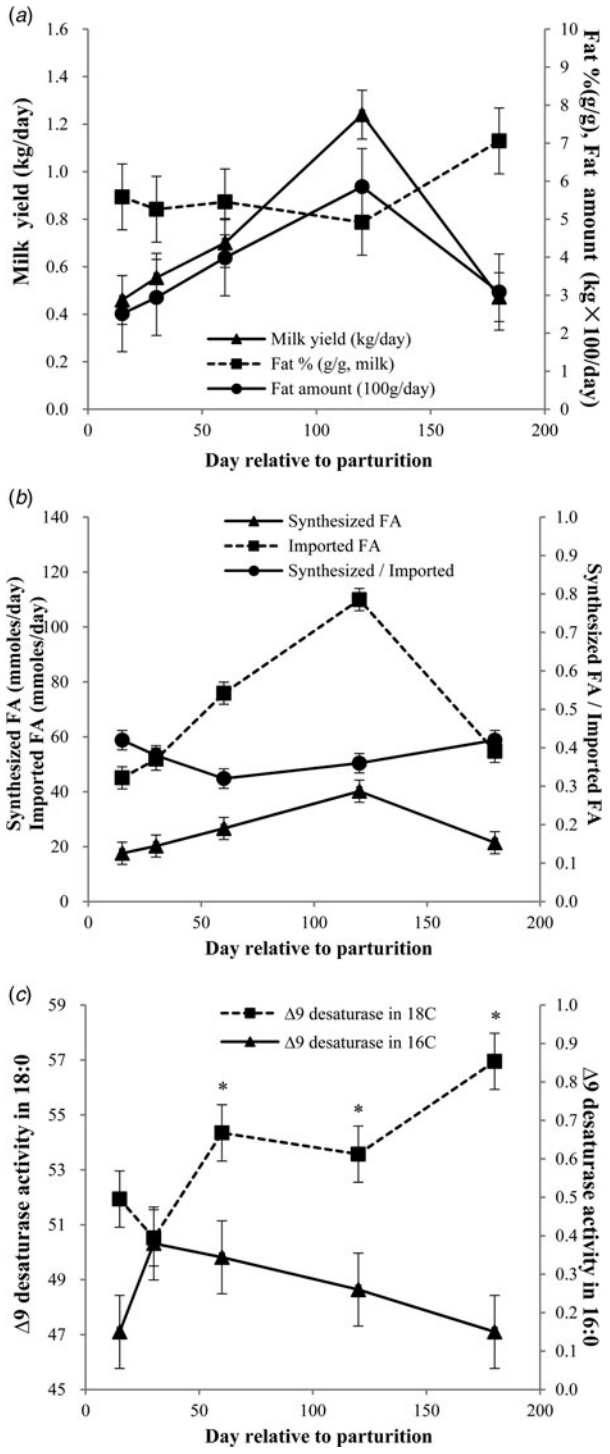

Fig. 1. Milk yield and the functional analysis of milk fat. (a) Yak milk yield, fat yield and fat % contents during lactation. Error bars indicate SEM. (b) Comparison of *de novo* synthesised FAs (mmoles/d) and imported FAs (mmoles/d) (*de novo* synthesised FAs were calculated based on FAs with C10–C14 as SCFAs, and imported FAs were estimated based on FAs with C18–C22 as LCFAs). Statistical effects with time; $P < 0{\cdot}05$ for milk yield; fat % did not change with time ($P = 0{\cdot}48$). Error bars indicate SEM. (c) Δ9 desaturase activity in C16 and C18. *Indicates that a point differs from the initial value. SEM was calculated (Supplementary Table S3).

increased with time during lactation and was produced at an amount equal to approximately 10% of that of dairy cows (0·4 ~ 1·2 kg/d in yak, Supplementary Table S3). The highest yield of yak milk occurred at 120 d ($P = 0{\cdot}003$). The yak is a seasonal animal, thus, in addition to the time relative to parturition, the yak's milk yield is dependent on the season. Our research was performed using yaks calving in May. In this case, the milk yield is known to be maximal at 90–120 d (Wiener et al. 2006). The fat concentration in yak milk did not differ with time ($P = 0{\cdot}394$), although the amount of fat produced significantly differed with time ($P = 0{\cdot}003$; Fig. 1a). LCFAs are primarily imported from blood to produce milk fat during the early lactation period, after which *de novo* FA synthesis increases in the inner mammary cell cytoplasm in lactating cows (Bionaz & Loor, 2008). However, milk fat production in lactating yaks differs from that in lactating cows, namely, the ratio of synthesised FAs to imported FAs did not effectively change ($P > 0{\cdot}05$), but synthesised FAs increased up to120 d ($P = 0{\cdot}005$, Fig. 1b).

Most of the genes related to milk synthesis were up-regulated during the yak lactation period

Most of the evaluated genes (80% of the surveyed genes related to milk fat synthesis) were up-regulated during the lactation period, and the expression levels of approximately 60% of the up-regulated genes significantly increased with time (Supplementary Table S5, Figs. S1–S4). The up-regulation of yak milk fat synthesis genes usually reached a maximum within 30 d, compared to the peak of 60 d in dairy cows. Among these genes, effective up-regulation ($P < 0{\cdot}05$) during lactation was observed in genes related to FA uptake from blood (*LPL, CD36*), intracellular FA transport (*FABP3*), intracellular FA activation of LCFA and SCFA (*ACSS1, ACSS2, ACSL1*), *de novo* synthesis (*ACACA*), desaturation (*SCD*), TAG synthesis (*AGPAT6, GPAM, LPIN1*), lipid droplet formation (*PLIN2, BTN1A1, XDH*), ketone body utilisation (*BDH1, OXCT1*), and transcription regulation (*THRSP, PPARGC1A*). As a result, the expression patterns of most of the yak milk fat synthesis genes and their % mRNA abundance levels were similar to those from dairy cows ($r > 0{\cdot}5$ in % mRNA abundance of all investigated genes; the correlations of the expression patterns in Supplementary Table S5).

Genes in de novo FA synthesis and TAG synthesis were more expressed and those in lipid droplet formation were less expressed in lactating yaks

Specifically, the relative % mRNA abundances levels of genes in *de novo* FA synthesis (*ACSS2*, *ACACA*, and *FABP3*) and TAG synthesis (*GPAM, AGPAT6,* and *LPIN1)* were comparatively prominent during the entire yak lactation period compared to those for dairy cows (>2-fold increase in % mRNA abundance compared to dairy cows, Fig. 2, Supplementary Table S5) and were up-regulated

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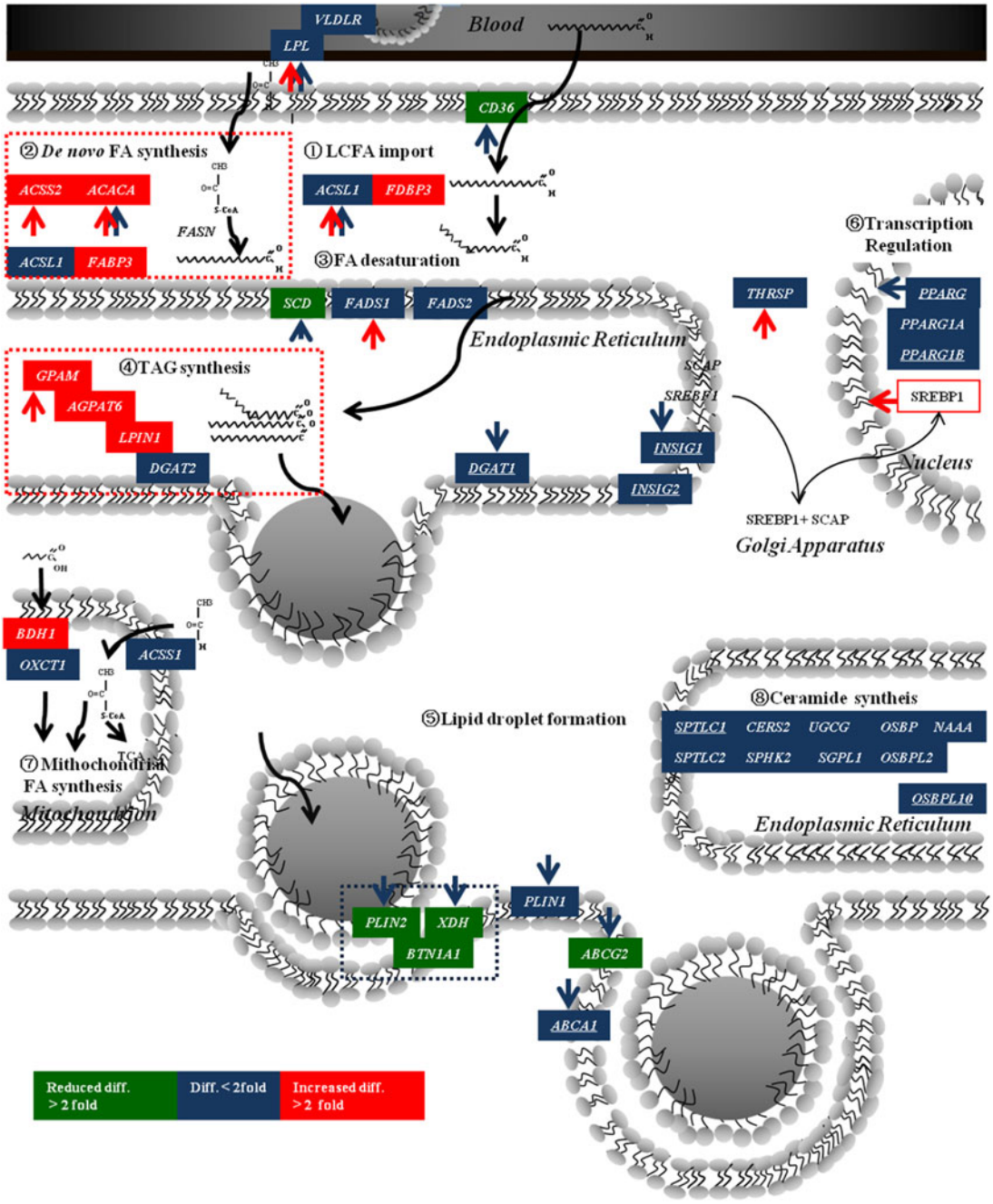

Fig. 2. Milk fat synthesis gene network in lactating yaks. The expression profiles of genes involved in lactating yak milk fat synthesis were investigated and compared to those of dairy cows using % mRNA abundance of all investigated genes (Supplementary Table S4). Genes with differences of % RNA abundance greater than 2-fold compared to those of dairy cows were marked with red boxes in cases of increased expression in yaks, and with green boxes in cases of reduced expression. Blue boxes indicate genes with differences in % RNA abundance of less than 2-fold or % mRNA abundance less than 1%. Underlined genes indicate negative correlation relationships in expression pattern during lactation with the corresponding dairy cow genes based on a Pearson correlation analysis using SAS (v 9.4, SAS Institute Inc. USA) (Supplementary Table S4). Gene networks were developed using Ingenuity Pathway Analysis (Ingenuity Systems, http://www.ingenuity.com, Supplementary Fig. S5) and Uniprot (http://www. uniprot.org/uniprot). Red and blue arrows indicate genes under the control of *SREBF1* and *PPARG*, respectively.

during early lactation within 5 d (Supplementary Figs. S1–S4). However, genes involved in FA desaturation, i.e., *SCD*, and lipid droplet formation (*BTN1A1*, *XDH*, and *PLIN2*) were expressed at relatively lower levels in yak mammary tissue (>2-fold decrease in % mRNA abundance compared to dairy cows, Fig. 2, Supplementary Table S5).

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The % mRNA abundance was similar pattern between all tested yaks (correlation value, $r > 0{\cdot}65$ ($P < 0{\cdot}05$) in all yaks, Supplementary Table S6).

The processes under the control of SREBP1 was more activated but those regulated by PPARG were less activated in lactating yaks

According to Bionaz & Loor (2008), *SREBF1* mRNA expression during lactation is thought to be central for *de novo* synthesis and TAG synthesis. In addition, *PPARG* is a main nuclear factor involved in the control of lipid droplet secretion and FA trafficking during lactation (Supplementary Fig. S5). Thus, two nuclear factors, *SREBF 1* and *PPARG*, have a role in controlling milk fat genes expression during lactation (Bionaz & Loor, 2008; Xu et al. 2016).

The precursor to SREBP1 binds to SCAP and is retained in the endoplasmic reticulum membrane. When INSIG1 is not present in the endoplasmic reticulum and cannot bind to SCAP, the SREBP1-SCAP complex can move to Golgi apparatus which contains proteases to degrade immature SREBP1 to a mature form, which is then transported to the nucleus to simulate FAs and cholesterol synthesis (Harvatine & Bauman, 2006). Thus, *INSIG1* expression negatively regulates *SREBF1* (Espenshade & Hughes, 2007). In our results, *INSIG1* was expressed at a lower level compared to that in dairy cows and had a negative relationship with dairy cow *INSIG1* expression ($r = -0{\cdot}62$) (Supplementary Table S4). Thus, we could suppose that the reduction of *INSIG1* expression induced SREBP1 to move into the nucleus and to activate the transcription of genes involved in *de novo* FA and TAG synthesis in lactating yaks. Therefore, the high fat contents in yak milk were assumed to be achieved *via* the stimulation of the processes of *de novo* FA and TAG synthesis by regulation under the control of SREBP1.

The genes related to lipid droplet formation in milk synthesis, such as, *BTN1A1*, *PLIN2*, and *XDH*, were up-regulated with time ($P < 0{\cdot}05$ in all three genes, Supplementary Fig. S2) but were expressed at comparatively lower levels compared to those in dairy cows, with a difference of greater than 2-fold (Fig. 2, Supplementary Table S5). The genes related to lipid droplet formation for milk synthesis are regulated by *PPARG* expression, which binds the *PLIN2* gene promoter and activates *PLIN2* expression in ruminant animals (Kang et al. 2015). PLIN2 protein in lipid droplet membranes sequentially binds to XDH and BTN1A1 to export fat to milk (Supplementary Fig. S5). *PPARG* was up-regulated 4-fold with time in lactating cows but was not significantly expressed with time in lactating yaks (Supplementary Table S5). The lower expression of the *PPARG* gene in lactating yaks was assumed to affect the transcription of *PLIN2*, which is a factor responsible for delivering the milk lipid droplet for transport.

Additionally, the expression profile of *SCD* reduction regulated by *PPARG* expression coincided with the results of FA composition. This finding is significantly different from that of dairy cows, in which *SCD* had the highest increase in the relative % mRNA abundance (23% in cows, 6·8% in yaks, respectively. Supplementary Table S5), although the patterns of *SCD* expression were similar in yaks and dairy cows ($r = 0{\cdot}8$). We also surveyed desaturase indexes as indicators of *SCD* activity by analysing the 16:0 and 18:0 FA compositions. Oleic acid (18:1, *n*-9) was compared to stearic acid (18:0); oleic acid comprised approximately 53% of the total 18-carbon FA in the lactating yak milk and increased with time ($P = 0{\cdot}001$, Fig. 1c). This result is 10% less than that in cows (approximately 63% in dairy cows) (Bionaz & Loor, 2008). The palmitoleic acid (16:1, *n*-9) content did not change with time in yak (Fig. 1c). *SCD* had comparatively lower expression and desaturation activity in lactating yaks than in dairy cows.

Conclusions

In our study, we investigated the difference in gene expression related to milk synthesis in lactating yaks compared to the expression profiles of lactating cows. Among milk fat synthesis processes, intracellular *de novo* FA synthesis (*ACSS2*, *ACACA,* and *FABP3*) and TAG synthesis (*GPAM*, *AGPAT6*, and *LPIN1*) were comparatively more activated, which might be accomplished in the orchestration of the gene networks under the control of *SERBP1* expression. In contrast, the genes involved in lipid droplet formation (*PLIN2*, *XDH*, and *BTN1A1* genes), which are mainly controlled by the regulator PPARG, were expressed at lower levels compared to those in dairy cows.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029917000413.

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