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ACACA, *FASN* and *SCD* gene expression in somatic cells throughout lactation and its relation to fatty acid profile in cow milk

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Acetyl-CoA Carboxylase α (*ACACA*), Fatty Acid Synthase (*FASN*) and Stearoyl-CoA Desaturase (*SCD*) genes encode key enzymes in fatty acid synthesis. The aim of this research was to verify if *ACACA*, *FASN* and *SCD* expression changes with lactation stages, and to analyze their expression levels and their relationship with the milk fatty acid profile. Milk samples were collected three times from each cow (n=11): at 50–100 DIM, 170–200 DIM and >250 DIM. RNA for gene expression analysis was extracted from milk somatic cells. *ACACA* expression was significantly greater at the 3rd compared to the 1st lactation stage. *FASN* and *SCD* expression significantly increased in the 3rd compared to the 1st and 2nd stages. The strongest positive, highly significant correlations were found in the 3rd lactation stage between *ACACA* and *FASN* expression, and between *FASN* and *SCD* expression. Few significant correlations were found between gene expression and fatty acid content in milk. Interestingly, no significant correlation was found between the *ACACA* or *FASN* genes and

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fatty acids synthesized *de novo*. No significant correlation was observed between the *SCD* gene and monounsaturated fatty acids. It was demonstrated that *ACACA*, *FASN* and *SCD* mRNA expression changes during lactation and is strongly coordinated. Poor correlation was found between fatty acid contents and gene expression.

KEY WORDS: cow milk / fatty acids / gene expression

It is well-known that the fatty acid profile in milk changes during lactation. In research conducted by Brainbridge *et al*. [2016] only two out of 40 analyzed fatty acids (FA) were unaffected (C18:2 *cis*-9, *cis*-12; C18:2 *cis*-9, *trans*-11) by lactation stage, while the other 38 were highly significantly affected by this factor. Similarly, Bastin *et al*. [2011] showed that levels of unsaturated fatty acids (UFAs), especially monounsaturated fatty acids (MUFAs), were affected by lactation stage after 100 days in milk(DIM). Additionally, the group of long-chained fatty acids (LCFAs) was less stable during lactation than the groups of medium-chain fatty acids (MCFAs) and short-chain fatty acids (SCFAs). It was also observed that the level of the C18 FA group (C18:0, C18:1, C18:1 *cis* and 18:1 *cis*-9) decreased during lactation. Gottardo *et al.* [2017] obtained comparable results indicating the SCFA group as the most stable across lactation, as well as the C18:0 level decreasing with lactation stage. In our previous research, the 18C group and all the other FA groups were also affected by lactation stage [Kęsek-Woźniak *et al*. 2020].

Due to the broad spectrum of action of fatty acids on the human body, it is important to thoroughly understand the molecular mechanisms regulating fatty acid synthesis in the bovine mammary gland. The Acetyl-CoA Carboxylase α (*ACACA*), Fatty Acid Synthase (*FASN*) and Stearoyl-CoA Desaturase (*SCD*) genes encode proteins (Acetyl-CoA carboxylase, Fatty Acid Synthase and Stearoyl-CoA Desaturase, respectively), which are key enzymes in fatty acid synthesis [Kęsek *et al.* 2014]. Recent findings have indicated genes differentially expressed in the mammary tissue during lactation [Yang *et al.* 2015, Cui *et al*. 2014]. Bionaz and Loor [2008] described a network of genes involved in FA synthesis and secretion, including *ACACA*, *FASN* and *SCD*, which were significantly upregulated throughout lactation. Moreover, many studies investigated the influence of several external factors such as different diets and supplementation on gene expression in the mammary gland [di Martino *et al.* 2015, Ibeagha-Awemu *et al*. 2016, Carraro *et al*. 2019].

The aim of the present study was to verify if *ACACA*, *FASN* and *SCD* expression changes with lactation stages, as well as analyze the relationship between *ACACA*, *FASN* and *SCD* expression and the fatty acid profile during lactation.

Material and methods

Animals and sample collection

Milk from Polish Holstein-Friesian cows (n=11), from the same farm and fed with the total mixed ration (TMR), were used in this study. All animals were healthy,

somatic cell count was <400 000. Milk samples were collected three times from each cow: at the 1st lactation stage (50-100 DIM), 2nd lactation stage (170-200 DIM) and the 3rd lactation stage (>250 DIM). Milk (400 ml/sample) was cooled to 4°C immediately after collection to minimize losses in somatic cell viability.

Somatic cell extraction and RNA isolation

Somatic cell count (SCC) was analyzed with the use of Somacount 150 (Bentley Instruments Inc., Chaska, MN, USA).

Milk somatic cells were obtained according to the methodology of Boutinaud *et al.* [2002] with modifications by Feng *et al*. [2007]. Milk from each sample was poured through a sterile filter and separated into glass centrifuge tubes, followed by the addition of chilled phosphate buffer (PBS) with 0.5 mM EDTA (pH=7.2). Milk was centrifuged at 2700 rpm for 10 min. at 4°C to remove the top layer of fat used for fatty acid profile analysis, while skimmed milk was used to extract somatic cells. The obtained pellets of somatic cells extracted from the bottom of the centrifuge tube were suspended in ice cold PBS. Then a series of at least five cycles of centrifugation and washing in ice cold PBS were performed (2700 rpm, 15 min, 4°C). After each centrifugation the supernatant was removed and fresh PBS was added. Before the last centrifugation the pellet was moved to a DNA-RNA free small test-tube. After removing the supernatant, purified pelleted cells were suspended in 1 ml of RNAlater (Sigma-Aldrich, St Louis, MO, USA) and snap frozen in liquid nitrogen. All samples were stored at -80°C until used.

From the extracted somatic cells total RNA was isolated using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Vilnus, LT), followed by the RNase-free DNase Set (Syngen Biotech, Wrocław, PL) treatment step to minimize genomic DNA contamination, according to manufacturer's instructions. The quantity and purity of RNA were measured using the NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The analysis of RNA integrity was performed by agarose gel electrophoresis.

Quantitative Real-Time PCR analysis

For cDNA synthesis, total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems by Thermo Fisher Scientific, Vilnius, LT). The PCR was carried out using an AriaMX Real-Time PCR System apparatus (Agilent Technologies, Santa Clara, CA, USA) in 96-well plates. Each qRT-PCR reaction (10 µl) contained 5 µl of SYBRTM Select Master Mix (Applied Biosystems by Thermo Fisher Scientific), a final concentration of 0.5 µM each of forward and reverse primers, 3 µl of DNase RNase free water and 1 µl of cDNA (100 ng). The thermal cycling program involved 50°C for 2 min (1 cycle), 95°C for 2 min (1 cycle), 95°C for 15 sec, 58°C for 15 sec, 72°C for 1 min (40 cycles). Primers for the housekeeping β-actin gene (*ACTB*) were designed using the Primer-Blast software [Ye *et al.* 2012] and verified using the Oligo Calc: Oligonucleotide Properties Calculator

Table 1. Gene abbreviation, gene name, primer sequences and sources

Gene abbreviation	Gene name	Primer sequences	Source
ACACA	Acetyl-CoA Carboxylase α	For: 5′-CATCTTGTCCGAAACGTCGAT-3′	Bionaz and Loor [2008]
		Rev: 5′-CCCTTCGAACATACACCTCCA-3′	
FASN	Fatty Acid Synthase	For: 5′-ACCTCGTGAAGGCTGTGACTCA-3′	Bionaz and Loor [2008]
		Rev: 5′-TGAGTCGAGGCCAAGGTCTGAA-3′	
SCD	Stearoyl-CoA Desaturase	For: 5′-TCCTGTTGTTGTGCTTCATCC-3′	Bionaz and Loor [2008]
		Rev: 5′-GGCATAACGGAATAAGGTGGC-3′	
ACTB	β-Actin	For: 5′-GATCTGGCACCACACCTTCT-3′	This manuscript
		Rev: 5′-CCAGAGGCATACAGGGACAG-3′	
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenase	For: 5′-CTTCAACAGCGACACTCA-3′	Zielniok *et al*. [2017]
		Rev: 5′-CCAGGGACCTTACTCCTT-3′	

[Kibbe 2007] to exclude sequences showing self-complementarity. Other genes were amplified using previously published primer sequences. All primers used for the reaction are shown in Table 1.

Each reaction was analyzed in triplicate. To determine the specificity of PCR products the dissociation curves were monitored, while negative and positive controls were run within each plate. The transcript levels were normalized to the *ACTB* and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase). The stability of these housekeeping genes was evaluated using the geNorm [Vandesompele *et al*. 2002] and BestKeeper software [Pfaffl *et al*. 2004].

Fatty acid profile analysis

The fatty acid profile was determined using a 7890 gas chromatograph with a flame ionization detector (Agilent Technologies), as described by Kęsek *et al.* [2017]. Individual FA esters were identified using a standard mixture of methyl-esters of fatty acids SupelcoTM 37 (Sigma Aldrich, St. Louis, MO, USA) and CLA *cis*-9, *trans*-11 and *trans*-10, *cis*-12 (Larodan, Malmö, Sweden), with the Agilent ChemStation software (Agilent Technologies).

Statistical analyses

Gene expression in lactation stages. Changes in relative concentrations were calculated using the RQMAX algorithm and converted into a $\log_2$ scale, according

to the method published previously [Livak and Schmittgen 2001, Smieszek *et al.* 2020]. Relative expression levels were compared using ordinary one-way analysis of variance (ANOVA) and Tukey's post hoc test. The data were analyzed using the GraphPad Software (Prism 8.20, San Diego, CA, USA). Results are presented as means ± SEM. Differences with a probability of $p<0.05$ were considered significant. Pearson's correlations between *ACACA*, *FASN* and *SCD* gene expression within individual lactation stages were calculated in JASP [JASP Team 2020].

Relationship between gene expression and FA profile. Desaturation indices (DI) for C14:1, C16:1 and C18:1 were calculated as follows: DI = *SCD* enzyme product/(*SCD* enzyme product + *SCD* enzyme substrate) [Rezamand *et al.* 2014].

Pearson's correlations between genes expression and the fatty acid profile and desaturation indices during each lactation stage were calculated in JASP [JASP Team 2020]. Results are presented as Pearson's correlation coefficient (r). Differences with a probability of $p<0.05$ were considered as significant.

Results and discussion

Gene expression in lactation stages

There was no difference in the *ACACA*, *FASN* and *SCD* gene expression between the 1st and 2nd stage of lactation. Levels of mRNA for *ACACA* (Fig. 1a) were not different in the 2nd compared to the 1st stage of lactation. However, this gene expression (Fig. 1a) was significantly ($p<0.05$) greater at the 3rd compared to the 1st stage of lactation. In contrast, mRNA expression for *FASN* (Fig. 1b) and for *SCD* (Fig. 1c) significantly increased in the 3rd compared to the 2nd stage, respectively, $p<0.001$ and $p<0.01$. Also, *FASN* (Fig. 1b) and *SCD* (Fig. 1c) expression was highly significantly ($p<0.001$) greater in the 3rd compared to the 1st stage of lactation.

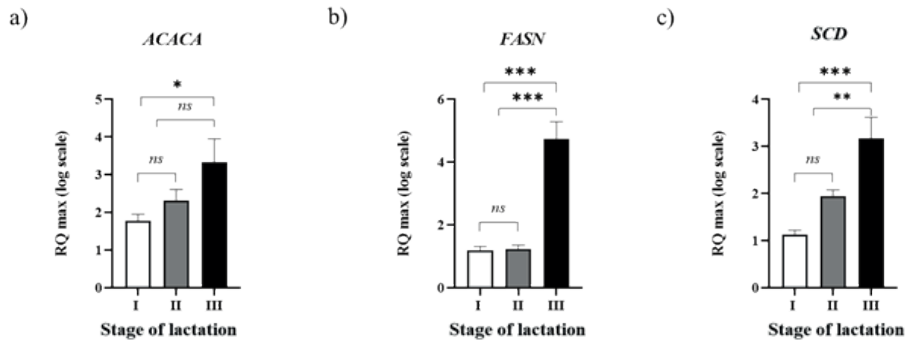

Fig 1. Quantitative real-time PCR analysis of *ACACA* (a), *FASN* (b) and *SCD* (c) gene expression relative to *ACTB* and *GAPDH* during 1st, 2nd and 3rd stage of lactation. Data are shown as means±SEM. Differences between lactation stages (i.e. 1st v 2nd; 1st v 3rd and 2nd v 3rd) are indicated as follows: ns – non-significant; * – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$.

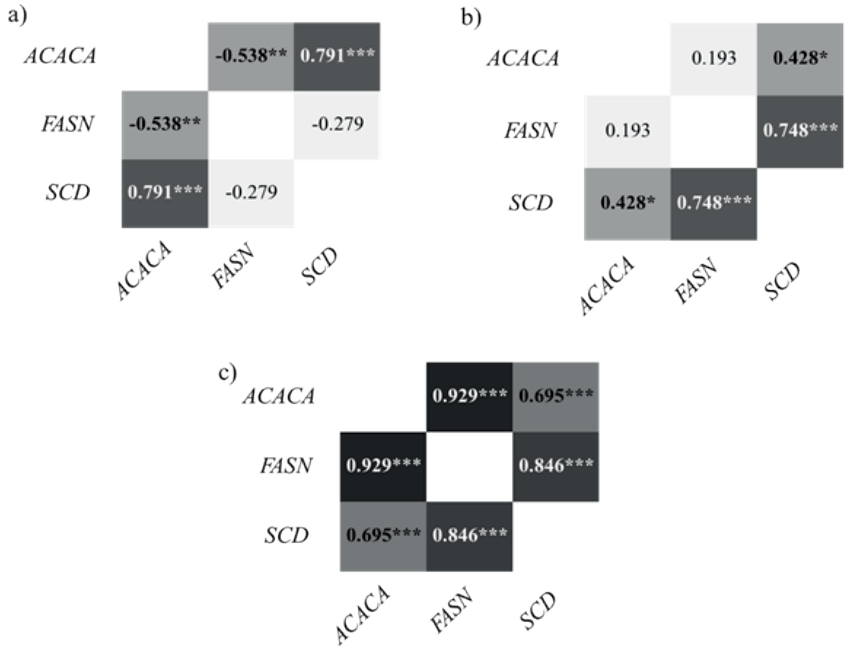

Fig. 2. Heatmap of Pearson's correlations coefficient between *ACACA*, *FASN* and *SCD* gene expression in 1st (a), 2nd (b) and 3rd (c) stage of lactation. Significant differences are indicated as follows: * – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$.

The strongest positive, highly significant correlations were found in the 3rd stage of lactation between *ACACA* and *FASN*, as well as between *FASN* and *SCD* (Fig. 2). Interestingly, the correlation between *ACACA* and *FASN* expression was significantly negative during the 1st lactation stage, weak and non-significant during the 2nd lactation stage and very strong positive, highly significant during the 3rd lactation stage. The correlation between *ACACA* and *SCD* expression remained significant and more constant between lactation stages. The correlation between *FASN* and *SCD* expression started as negative and non-significant, to become strongly positive and highly significant during the 2nd and 3rd lactation stages.

Bionaz and Loor [2008] analyzed mRNA abundance changes relative to lactation day -15 of several lipogenic genes. Those authors demonstrated a greater up-regulation of *ACACA* expression compared to *FASN* expression during lactation, despite the fact that *ACACA* mRNA accounted only for <1%, while *FASN* mRNA accounted for 7% of the total genes measured. This higher up-regulation of *ACACA* mRNA is consistent with the activity values of the encoded enzymes [Mellenberger *et al.* 1973]. Bionaz and Loor [2008] emphasized that the expression patterns of both genes were analogous. In the present study, strong significant correlations between *ACACA* and *FASN* and *SCD* mRNA expression were observed, especially during the 3rd lactation stage. The highest abundance of *ACACA* and *FASN* expression was observed at 60 relative to -15

day, which is consistent with levels of synthesized fatty acids. Results for fatty acid changes during lactation obtained in this study (data not shown) are consistent with those reported by Bionaz and Loor [2008]. However, as in the presented study, the expression levels were normalized in relation to housekeeping genes, it is difficult to compare the obtained results with the findings of Bionaz and Loor [2008].

Relationship of gene expression and FA profile

Few significant correlations were found between gene expression and only some fatty acid content: for the *ACACA* gene C18:2 ω-6 cis and C14:1; for the *FASN* gene CLA and C20:4 ω-6; for the *SCD* gene C14:1, C18:2 ω-6 cis and C18:3 ω-3 (Fig. 3). Interestingly, no significant correlation was found between the *ACACA* or *FASN* genes, which encode enzymes involved in *de novo* synthesis of fatty acids chain except for

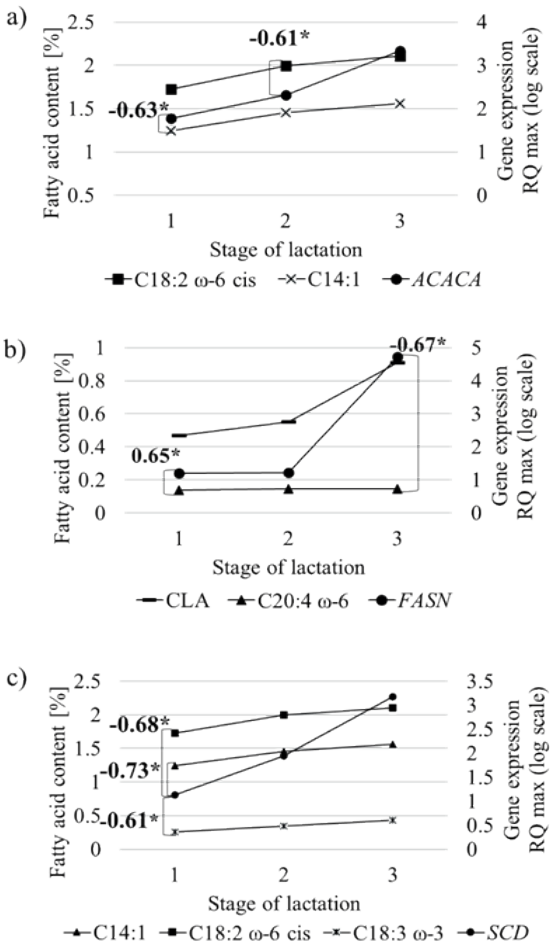

Fig. 3. Correlation analysis of *ACACA* (a), *FASN* (b), *SCD* (c) gene expression and fatty acid content during 1st, 2nd and 3rd lactation stage. Data are shown as means with significant Pearson's correlation coefficients. Significant differences are indicated as * – $p<0.05$.

C14:1 during the 1st stage of lactation with the *ACACA* gene. Regarding desaturation, the only significant correlation was observed between *FASN* gene expression and DI for C14:1 ($r = +0.654$; $p<0.05$) during the 2nd lactation stage.

It is important to emphasize that the fatty acid profile in milk is regulated by many factors, both external and internal [Kęsek *et al.* 2014], such as lactation stage, gene polymorphism [Kęsek-Woźniak *et al.* 2020] and diet supplementation [Bodkowski *et al.* 2020]. Also, gene expression is known to be regulated by external factors, such as nutrients, hormones, growth factors, as well as internal control mechanisms [Loor *et al.* 2022, Shao *et al.* 2013]. As shown by Bernard *et al*. [2008], *ACACA* and *FASN* expression in the mammary gland was found to be positively related with the C4:0 to C16:0 yield after dietary treatment, which suggests that changes in the expression of these genes are important factors in the dietary regulation of FA *de novo* synthesis. This dietary regulation of mRNA abundance related to FA contents can be explained by a supply-demand relation – the more FAs in the milk-producing cells are diet-derived, the lesser amounts of FAs have to be *de novo* synthesized. Lashkari *et al.* [2020] showed that diet supplementation influences mRNA abundance for *ACACA*, *FASN* and *SCD*, which was reduced with crushed sunflower seed supplementation. This reduction of *FASN* and *SCD* transcription reflected the reduction in FA *de novo* synthesis observed in the supplementation groups. Those authors also demonstrated that diet-induced down-regulation of *ACACA* and *FASN* transcription was coordinated. In the present study a correlation between *ACACA* and *FASN* mRNA levels was also observed, which suggests a coordinated expression of these two genes.

The *SCD* enzyme activity is very important for milk fat synthesis in ruminants, as the mammary gland absorbs mainly SFAs due to rumen biohydrogenation [Rezamand *et al.* 2014]. Bionaz and Loor [2008] observed the highest quantity of *SCD* mRNA, as well as its strongest (over 40-fold) up-regulation during lactation compared to the other analyzed genes. Interestingly, no significant correlation was observed between the *SCD* gene and monounsaturated fatty acids, except for C14:1 during the 1st lactation stage in the present study. The correlation between the *SCD* gene expression and DI varies between different studies. Rezamand *et al.* [2014] found a positive correlation between *SCD* gene expression and desaturase indices for C18:1, CLA and the sum of all these indices. This is in contrast to the presented study (no correlation observed), as well as the results obtained by Bionaz and Loor [2008], who did not detect any correlation, or Archibeque *et al*. [2005], who detected only a poor association.

In summary, the expression of the *ACACA*, *FASN* and *SCD* genes, which are involved in fatty acid synthesis, changes during lactation and is strongly coordinated. Poor correlations were found between fatty acid contents and mRNA expression of *ACACA*, *FASN* and *SCD*. However, it is important to remember that fatty acid synthesis in the mammary gland is a very complex process, regulated by many factors, especially by dietary treatments.

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