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Nutritional modification of *SCD*, *ACACA* and *LPL* gene expressions in different ovine tissues

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Abstract. Fatty acid composition is one of the main factors affecting health benefits of food. Stearoyl-CoA desaturase 1 (*SCD*), acetyl-CoA carboxylase alpha (*ACACA*) and lipoprotein lipase (*LPL*) have been considered as the rate-limiting enzymes in the biosynthesis of different fatty acids critical in lipid metabolism. The aim of our study was the analysis of differences in expression profiles of three ovine genes related to lipid metabolism (*LPL*, *ACACA*, *SCD*) depending on feeding system and tissue type. The gene expression measurement was performed using a real-time PCR method on 60 old-type Polish Merino Sheep, which were divided into three feeding groups (I – complete pellet mixture, $n = 12$; II – complete mixture with addition of fresh grass, $n = 24$; III – complete mixture with addition of fresh red clover, $n = 24$). From all lambs, tissue samples – subcutaneous fat, perirenal fat and liver – were collected immediately after slaughter and *LPL*, *ACACA* and *SCD* expression was estimated based on two endogenous controls (*RPS2* – ribosomal protein S2; *ATP5G2* – H(+)-transporting ATP synthase). Our research indicated that supplementation of diet with an addition of fresh grass or red clover significantly ($P < 0.05$) decreased the expression of *SCD*, *ACACA* and *LPL* genes in fat tissue compared to standard complete pelleted mixture. On the other hand, the highest expression of *ACACA* was detected in liver tissue collected from sheep fed a diet with an addition of fresh red clover ($P < 0.05$). In turn, the highest expression of the *SCD* gene was detected in animals fed with grass supplementation ($P < 0.05$). Regardless of diet supplementation, the highest *SCD* transcript abundance was detected in perirenal fat, while *LPL* and *ACACA* expression was the highest in both perirenal and subcutaneous fat. The ability of nutrigenomic regulation of transcription of analyzed genes confirmed that these genes play a critical role in regulation of lipid metabolism processes in sheep and could be associated with fatty acid profiles in milk and meat.

1 Introduction

In sheep production, an increase and/or maintaining a satisfactory level of proper ratio of polyunsaturated (PUFAs) to saturated fatty acids (SFAs) in dairy products and meat (Wood et al., 2003) have become of the greatest importance. The modification of the fatty acid profile has been intended to increase the content of omega-3 fatty acids and conjugated linoleic acid (CLA) and is usually obtained by diet supplementation. On the other hand, diet supplementation with expensive additions significantly increases costs of animal production, which can become unprofitable. It has been confirmed that modification of the expression of genes involved in lipid metabolism is associated with a change in lipid profiles in ovine tissues such as skeletal muscle, cardiac muscle and adipose tissue (Bonnet et al., 2000). Thus, due to the health-promoting properties of sheep products and relatively

high costs of different diet supplements, research has been performed to establish genetic markers related to fatty acid composition.

The stearoyl-CoA desaturase 1 (*SCD*), acetyl-CoA carboxylase alpha (*ACACA*) and lipoprotein lipase (*LPL*) have been considered as the rate-limiting enzymes in the biosynthesis of different fatty acids critical in lipid metabolism. The SCD enzyme plays a key role in synthesis of monounsaturated fatty acids (MUFAs) from saturated fatty acids (SFAs; Ntambi et al., 2002), LPL participates in triglyceride-rich lipoprotein metabolism, and ACACA regulates biosynthesis of palmitic acids and long-chain fatty acids. All these enzymes are expressed ubiquitously throughout different tissues, but the highest expression has been detected in adipocyte tissue, liver and also in mammary glands during lactation (Jensen et al., 1991). Is has been established that the activity of the ACACA enzyme is associated with palmitic acid content in milk, which is considered as undesirable component increasing the risk of occurrence of cardiovascular diseases (Fattore and Fanelli, 2013). In turn, the *SCD* enzyme is responsible for biosynthesis of conjugated linoleic acid (CLA, isomer *cis*-9, *trans*-11), which is also secreted in milk and shows many beneficial effects on human health. Moreover, the ovine *SCD* gene is localized on chromosome 22 within a QTL (quantitative traits locus) region associated with the ratio of CLA to vaccenic acid in milk (Carta et al., 2008). The hydrolysis of the triacylglycerol (TAG) component of chylomicrons and the very low-density lipoprotein fraction of lipoproteins is catalyzed by LPL enzymes, which are also the primary source of long-chain fatty acids taken up by the mammary gland (Bernard et al., 2008). According to the function of LPL, ACACA and SCD enzymes, their genes are examined as candidate markers related to fatness traits in sheep.

Thus, the aim of our study was the analysis of differences in expression profiles of three genes related to lipid metabolism depending on feeding system and tissue type. Many reports show that the *LPL*, *ACACA* and *SCD* genes can be regulated by nutrition, but the exact mechanism of such modifications is still unknown. The obtained results will be useful to identify the molecular basis of lipid metabolism in ovine tissues and can be a base for further research focusing on determination of genes involved in fatty acid composition in milk and meat.

2 Material and method

2.1 Animals

The analysis was performed on 60 old-type male Polish Merino Sheep. All animals were maintained in the Experimental Station of the National Research Institute of Animal Production in Pawłowice under the same housing conditions. Lambs were divided into three feeding groups according to the system of feeding ad libitum with isoenergetic diets:

i. complete pelleted mixture according to INRA norm (IZ PIB-INRA, 2009, $n = 6$);

ii. complete mixture with addition of fresh pasture grass ($n = 12$);

iii. complete mixture with addition of fresh red clover ($n = 12$).

The detailed composition and nutritional value of complete mixture for lambs control group (diet I) and for experimental groups (diets II and III) were presented in Tables S1 and S2 in the Supplement. In diets II and III, the complete mixture was administered in an amount of 3 % of body weight, while supplementation of fresh grass/red clover was 3 kg per lamb administered after concentrates intake. To balance dietary fiber, animals received meadow hay as a supplement (100 g sheep^{-1} day^{-1}). The weight of refusals was daily controlled. The diets were formulated using a feed optimizing software tool (Zifo WIN v1.5; 2012). The composition and nutritional value of each diet and the exact feed conversion ratios (FCRs; kg kg^{-1}) according to feeding system were presented in Table 1.

The feeding experiment was performed in a duplicate (the repetitions of experiment were performed within two years) in the same scheme and included 30 animals for each replicate. Animals were maintained in group pens (six lamb per pen) according to feeding system. Lambs were fattened from an average of 28 kg to a final body weight of 41.5–43 kg (day at slaughter was from 170 to 195, an average of 183 days), slaughter and dissected. From each animal, immediately after slaughter, tissue samples (liver, subcutaneous fat, perirenal fat) were collected into tubes with RNAlater® solution (Ambion, Thermo Fisher Scientific, USA) and stored at −20 °C. For fatty acids composition analysis, the longissimus dorsi muscle samples behind the last rib were collected and then stored at −20 °C. Muscle for expression measurements and fatty acid analysis was sampled from the same part of the longissimus dorsi muscle.

2.2 Genes expression measurement

Isolation of total RNA was carried out in total for 180 samples using a PureLink™ RNA Mini Kit (Ambion, Thermo Fisher Scientific, USA) according to the protocol. The quantity of the obtained RNA was estimated using a NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, USA), while RNA degradation was checked on 2 % agarose gel. The cDNA was obtained from 250 ng of total RNA using a Maxima First Strand cDNA synthesis kit for RT-qPCR (Thermo Scientific) according to the attached protocol. The gene expression was measured on a 7500 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific) using the TaqMan® Gene Expression Master Mix (Applied Biosystems, Thermo Fisher Scientific), primer sets and TaqMan probes. Primers and probes for target genes were de-

Table 1. The composition and nutritional value of each diet and the exact feed conversion ratios.

	Feeding groups		
	Diet I – complete mixture $n = 6$	Diet II – grass addition $n = 12$	Diet III – red clover addition $n = 12$
Composition of integrities, kg:			
complete mixture	1.65	0.92	0.95
meadow hay	0.08	–	–
meadow grass	–	2.37	–
red clover	–	–	2.44
Composition of integrities in the ration, kg:			
metabolizable energy, MJ	17.0	14.0	13.6
total protein, g	313.5	242.7	250.6
useful protein nBO, g	262.4	201.2	202.9
protein UDP, %	28.2	24.7	22.1
crude fat, g	43.2	39.4	37.9
ash content, g	133.3	109.8	113.4
crude fiber, g	144.4	133.4	121.1
calcium, g	19.3	15.9	18.8
phosphorus, g	8.4	6.9	6.7
sodium, g	6.4	2.7	2.9
magnesium, g	3.5	3.4	3.6
Fatty acid concentration (presented as % of total fatty acid content)			
linoleic acid (C18:2)	16.2	12.8	12.8
polyunsaturated fatty acid n-3 + n-6	18.6	16.0	15.7
feed conversion ratio (FCR) per 1 kg of gain	5.14	3.34	3.29
Feed unit for maintenance and meat production UFV	4.16	5.56	5.73

signed using Primer Express 3.0 software, while primers and probes for the two endogenous controls were synthesized by Primerdesign (Primerdesign, Southampton, UK) as a custom-designed real-time PCR assay with a double-dye probe and primer-limited concentration (Table S3). Relative quantification was performed in 45 cycles, for each sample in three replications, and multiplexed as follows: *LPL* and *ATP5G2*; *SCD1* and *RPS2*. For the *ACACA* gene, reactions were performed without multiplexing. Two genes were used as endogenous controls (*RPS2* – ribosomal protein S2; *ATP5G2* – H(+)-transporting ATP synthase; Ropka-Molik et al., 2016). The reaction temperature steps were as follows: UDG incubation (uracil-DNA glycosylase treatment) – 50 °C (2 min); AmpliTaq Gold, UP enzyme activation – 95 °C (10 min); and denaturation (95 °C; 15 s) and annealing/extending (60 °C; 1 min) in 45 PCR cycles.

For each analyzed gene, the PCR efficiency was estimated based on the standard curve method and the exact transcript abundance was calculated using the $\Delta\Delta$ Ct method $(1/E(\mathrm{Ct}))$, where E is efficiency (10[−1/slope]) and Ct is cycle determined by the threshold applied to the maximum amplification of the standard curve. The sample with the lowest expression level was used as a calibrator in each gene separately. The normality of the distribution of expression data was assessed with the use of the Shapiro–Wilk test (SAS v. 8.02), while the differences in genes expression levels between tissues were analyzed using the Kruskal–Wallis test.

2.3 Estimation of fatty acid content in longissimus dorsi muscle

The fatty acid profile of the meat samples (longissimus dorsi muscle) was estimated by gas chromatography according to the method of Folch et al. (1957). The analysis was performed on a basis of fat extraction mixture composed of methanol and chloroform in a 1 : 2 ratio and then the residue was saponified with NaOH. Then, the formed methyl esters of fatty acids were examined using gas chromatography on a Varian 3400 (Sugar Land, TX, USA) analyzer, an 8200 CX injector and a flame ionization detector (FID). Chromatographic separation was performed on a CP Wax 58 column (0.53 mm × 1 µm; Chrompack, USA) using different temperature programs ranging from 60 to 188 °C (4 °C change

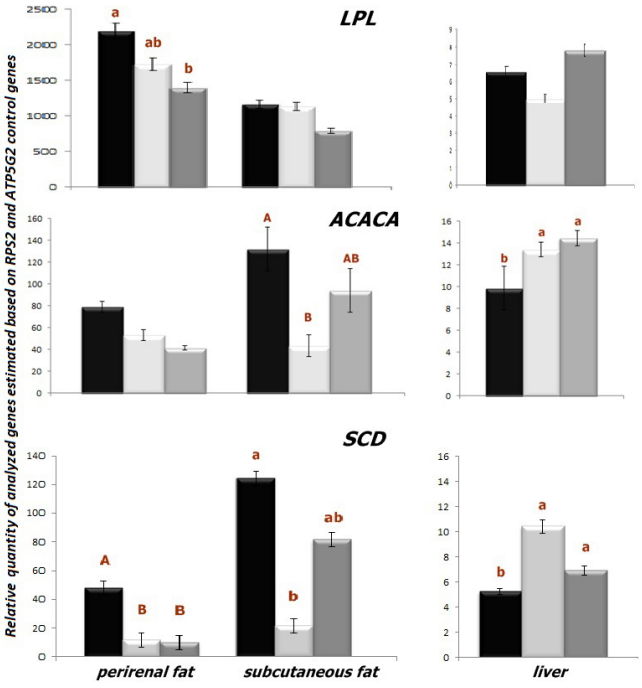

Figure 1. The transcript level for *LPL*, *ACACA* and *SCD* genes estimated in fat and liver tissues according to diet supplementation: complete pellet mixture (black column), grass addition (while column) and red clover addition (gray column). Data are presented as mean ± standard error; means with a, b and A, B differ significantly at $P < 0.05$ and $P < 0.01$, respectively.

per min) and then followed by temperature change from 60 to 220 °C (5 °C change per min). Injector and FID temperature was 200 and 260 °C, respectively. In addition, helium was used as a carrier gas (6 mL was added each minute). Hexane sample solutions (1 µL) were injected onto the column. The analyses were performed using standard solutions containing a mixture of standards (0.02–3.3 mg mL^{-1} in hexane) all purchased from Sigma-Aldrich (USA). Final results were adjusted for fatty acid content in a blank sample, which was prepared in a similar manner to the sample but without a weighed sample. Fatty acid content was expressed as percent of total fatty acids. The quantitative analysis of CLA isomers was performed by gas chromatography attached to Shimadzu GC-MS QP-2010 Plus mass spectrometry apparatus.

The significance of differences in fatty acid content between feeding groups were estimated using ANOVA with the Duncan post hoc test (SAS v. 8.02).

3 Results

3.1 Effect of the diet supplementation on transcript abundance of LPL, ACACA and SCD1

Our results showed that the expression of the *LPL* gene in perirenal fat tissue was modified by dietary supplement. Significantly ($P < 0.05$) higher transcript abundances were detected in perirenal fat tissue of sheep fed with pelleted complete mixture without the addition of grass/clover as a supplement, while the lower expressions were observed in animals fed with fresh red clover and supplemental mixture (Fig. 1). A similar trend was observed in subcutaneous fat, but without statistical significance.

The supplementation of diet with an addition of grass or red clover significantly ($P < 0.05$) decreased the expression of the stearoyl-CoA desaturase 1 gene in both fat tissues compared to standard pelleted complete mixture. In subcutaneous fat, the *SCD1* expression in sheep fed with a complete mixture was 5.75-fold higher than in animals fed with grass supplementation and 1.52 than in animals fed with an addition of red clover (Fig. 1).

The present research also showed a lower transcript level of the *ACACA* gene in subcutaneous fat of sheep fed with grass compared to standard pellet mixture ($P < 0.01$), similar to the case of the *SCD1* gene (Fig. 1). Intermediate values were obtained for the subcutaneous fat of animals which had their diet supplemented with fresh red clover.

For the *LPL* gene, we did not observe any significant differences in liver tissue regardless of diet supplementation. For *ACACA* the highest expression was detected in liver tissue collected from sheep fed a diet with an addition of fresh red clover (Fig. 1; significant for the *ACACA* gene; $P < 0.05$). In the liver, the highest expression of the *SCD* gene was detected in animals fed with grass supplementation ($P < 0.05$).

3.2 The expression level of *LPL*, *ACACA* and *SCD1* in different tissues

When comparing gene expression levels between different tissues in each feeding group, the highest amount of mRNA for all investigated genes was detected in fat, especially in perirenal fat. Regardless of diet supplementation, the highest *SCD* expression was detected in perirenal fat (Fig. 2), while *LPL* and *ACACA* transcript abundances were the highest in both perirenal and subcutaneous fat (Fig. 2). The lowest expression of all genes was detected in the liver.

3.3 Fatty acid composition following diet supplementation

Our results showed that meat (longissimus dorsi muscle) of lamb fed with fresh red clover and grass addition was characterized by significantly the highest SFA concentration as well as the highest total conjugated linoleic acid (CLA) content (Table 2). Furthermore, the highest concentration of c9-t11CLA was obtained for animals fed with red clover, while the lowest was found for lambs fed with the complete mixture ($p < 0.05$). For the rest of the isomers (CLA t10-c12, CLA c9-c11, CLA t9-t11) significant differences were not found. Moreover, the highest n-6 to n-3 polyunsaturated fatty acid ratio was detected in lambs fed with the standard com-

Table 2. The differences in selected fatty acid content estimated in lamb longissimus dorsi muscle depending on feeding system. Fatty acid contents are presented as g 100 g^{-1} of total fatty acids in muscle samples.

Trait	Feeding groups		
	Complete mixture (diet I)	Grass addition (diet II)	Red clover addition (diet III)
Back fat of saddle (kg)	0.167 ± 0.05a	0.102 ± 0.03b	0.139 ± 0.05ab
SFA	46.07 ± 1.37b	49.54 ± 2.43a	48.39 ± 2.57a
MUFA	43.15 ± 5.59	41.45 ± 3.53	42.20 ± 1.44
PUFA	10.77 ± 4.87	8.99 ± 2.28	9.39 ± 1.77
n-6:n-3 PUFA	12.89 ± 4.22a	8.24 ± 2.16b	6.67 ± 1.06b
CLA	0.228 ± 0.05b	0.361 ± 0.10a	0.325 ± 0.09a
c9t11CLA	0.115 ± 0.03c	0.190 ± 0.06b	0.254 ± 0.05a
18:1cis 9	13.42 ± 1.37	14.39 ± 1.80	13.20 ± 0.95
18:2n 6	12.42 ± 1.37	13.40 ± 1.80	12.21 ± 0.95
18:3n 3	11.42 ± 1.37	12.40 ± 1.80	11.21 ± 0.96

SFA – saturated fatty acid; MUFA – monounsaturated fatty acid; PUFA – polyunsaturated fatty acid; CLA – conjugated linoleic acid; c9t11CLA – *cis*-9, *trans*-11 CLA isomer; a, b, c – $P < 0.05$.

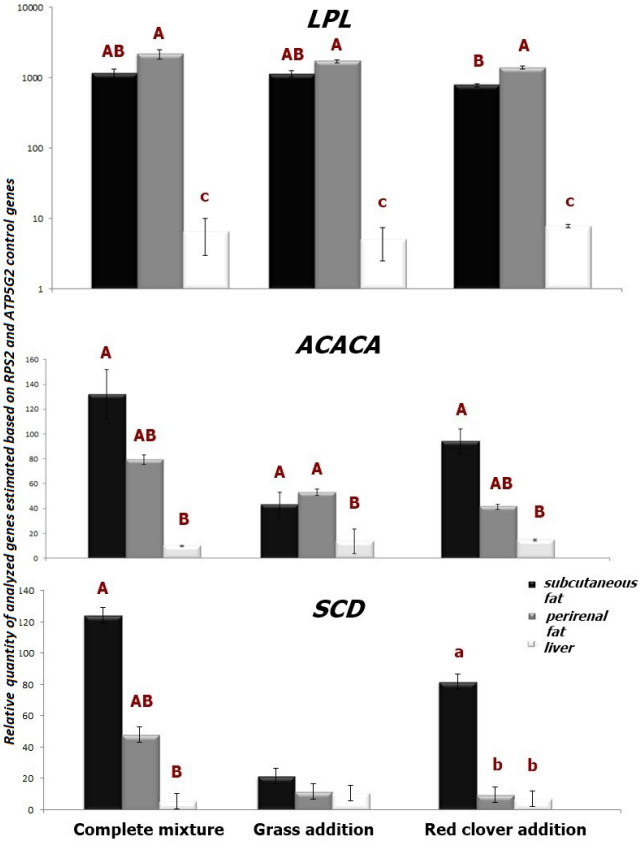

Figure 2. The differences in expression of *LPL*, *ACACA* and *SCD* genes between analyzed ovine tissues regardless of feeding supplementation. Data are presented as mean ± standard error; means with a,b and A,B differ significantly at $P < 0.05$ and $P < 0.01$, respectively.

plete mixture (which contains a higher concentration of carbohydrates compared to the other two feeding groups). The feeding system also affected the degree of fatness – lambs fed with complete mixture (diet I) had significantly higher back fat on the saddle compared to animals fed with fresh grass addition.

4 Discussion

Fatty acid composition is one of the main factors affecting health benefits of food. The ratio of polyunsaturated and monounsaturated to saturated fatty acid and the concentrations of omega-3 acids and conjugated linoleic acid (CLA) are considered as essential diet elements which may affect the prevalence of many diseases in society such cardiovascular disease, diabetes and cancer (Kritchevsky et al., 2000; Diniz et al., 2004; Liao et al., 2010). The main aim of the present research was to estimate the potential effect of diet supplementation on the transcript level of *LPL*, *ACACA* and *SCD* genes, which play a key role in lipid metabolism and adipogenesis process. Our studies are based on relatively affordable and accessible feeding supplements (grass and red clover addition to standard pellet mixture), which could potentially affect the expression of enzymes that are critical in fatty acid biosynthesis and/or hydrolysis of lipoprotein triacylglycerols. Previous research has confirmed that such nutritional regulation might result in modification of fatty acid composition, but the exact molecular mechanism of observed variations is still unknown (Murphy et al., 1999; Bonnet et al., 2000; Ntambi et al., 2004; Corazzin et al., 2013; Kęsek et al., 2014).

Our study showed that diet with an addition of fresh grass or red clover to standard pellet mixture significantly reduced the transcript levels of the *LPL* gene in perirenal fat, *ACACA* in subcutaneous fat and *SCD* in both fat tissues. In the ru-

men, unsaturated fatty acids have a short half-life according to their rapid hydrogenation to saturated fatty acids (Jenkins, 1993). It has been established that enrichment of the diet with pasture (grass or fresh red clover) results in greater fodder intake by animals due to the lower energy content in the diet. This leads to decreasing of residence time of forage in the rumen and results in reduction of hydrogenation process for some PUFAs. Moreover, the addition a red clover, which contains the polyphenol oxidase (PPO) enzyme, decreases proteolytic and lipolytic processes in the rumen and can reduce fatty acid biohydrogenation (Sullivan and Hatfield, 2006). Steinshamn and Thuen (2008) confirmed that the addition of red clover to standard diet leads to significant modifications of milk fatty acid profiles: an increase in C18:3n-3 and C18:2n-6 content and the reduction in n-6 / n-3 ratio. Our results indicated that feeding supplementation modified the expression of ovine *LPL*, *ACACA* and *SCD* genes in fat tissues as well as changed selected fatty acid profiles in lamb. Raes et al. (2004) showed that c9t11CLA content can be increased by feeding ruminants n-3-rich diets. In our study, it has been confirmed that diet with the high concentration of carbohydrates (complete mixture) led to the meat n-6 / n-3 ratio increasing by about 2 times compared to diet with red clover addition. Furthermore, diet supplementation with fresh grass or red clover increased total CLA content and c9t11CLA isomer concentration.

In the case of lipoprotein lipase, which is related to fat deposition in adipocyte tissue and synthesis of the low-density lipoprotein fraction, the addition of red clover reduced the *LPL* gene expression in perirenal fat. Intermediate values of transcript abundances were obtained for fat tissue of sheep fed with a grass addition. Research performed on chickens showed that a diet rich in n-3 and n-6 fatty acids reduced *LPL* expression measured in adipocytes compared to n-9 fatty acid addition (Montalto and Bensadoun, 1993; Sato and Akiba, 2002). On the other hand, in rats, a diet rich in n-3 acids increased both LPL activity and transcript levels in adipocyte tissue (Chapman et al., 2000). Furthermore, the *LPL* expression can be up-regulated by refeeding, which was established in cattle (Bonnet et al., 1998) and sheep (Bonnet et al., 2000).

The present study shows that transcription of the *LPL* gene in sheep was most efficiently modified by diet supplemented with red clover, probably due to the highest concentration of polyphenol oxidase. In the rumen, the PPO enzyme regulates the lipolysis and is critical for deposition of PUFA in animal products (Lee, 2014). Similarly to research performed by Dervishi et al. (2011), we detected the highest *LPL* expression in sheep fed an intensive diet. Interestingly, in the liver, the highest *LPL* expression, but without statistical significance, was observed in sheep fed with red clover, in contrast to the perirenal fat tissue. This reverse trend in the modification of *LPL* expression by different diet additions might be related to tissue-specific ability of distribution and synthesis of fatty acids and/or secretion of triglyceride-rich lipoproteins (Pullen et al., 1990). The tissue-specific regulation of *LPL* expression was also confirmed in rats, where a dietary content of n-3 PUFA affected *LPL* transcription in internal adipose tissue while lacking influence in subcutaneous adipose (Raclot et al., 1997).

Our study indicated that the transcript level of the *ACACA* gene might be regulated in a similar manner to the *LPL* gene. The feeding of sheep with grass or red clover significantly decreased expression of acetyl-CoA carboxylase alpha in subcutaneous fat ($P < 0.05$) and perirenal fat (without significance), while in the liver both supplements activated *ACACA* transcription ($P < 0.05$). Similar to the case of the *LPL* gene, the highest *ACACA* expression in the liver was detected in sheep fed with an addition of fresh red clover and grass. The ACACA catalyzed malonyl-CoA synthesis from acetyl-CoA and is mainly expressed in lipogenic tissues (Lopez-Casillas et al., 1991). Mao et al. (2003) showed that in, human transcription, the *ACACA* gene is regulated by three promoters and one of them is expressed in a tissue-specific manner. The adverse regulation of the ovine *ACACA* gene in the liver and fat in response to a different diet would suggest that, in sheep, this gene might have a different role in both tissues. Furthermore, in the liver and fat tissue different lipid metabolism processes occur that are related to modifications of distinct fatty acids which may also impose tissue-specific expression.

In turn, the results obtained showed that SCD expression in sheep fat tissue was down-regulated in response to a diet with an addition of grass and red clover. On the other hand, forage with fresh grass supplementation significantly increased SCD transcript levels in the liver. It has been proven that, in the most of tissues, a high-carbohydrate diet shows large stimulatory effects on the levels of SCD mRNA (Miyazaki et al., 2002). In mice, dietary intake of n-3 fatty acids, which is considered a strong SCD repressor, showed the inhibitory effect of stearoyl-CoA desaturase 1 expression in the liver, despite supplementation with carbohydrates (Ntambi, 1992, 1999). Our results indicated that an increase in intake of unsaturated fatty acid reduced *SCD* gene expression in ovine fat tissue but at the same time increased transcript level in the liver. Inversely, in both fat tissues of sheep fed with standard pellet mixture, which contains higher concentration of carbohydrates compared to two other forages, the detected expression of the *SCD* gene was higher, while in liver tissue it was lower. This suggests that in sheep transcription of the stearoyl-CoA desaturase 1 gene is modified in a tissue-specific manner, probably due to different metabolic activity of investigated tissues. It can also be related to fatty acid modification occurring in the rumen as well as diverse fatty acid composition and concentration which is transported to the liver and fat. The different regulatory mechanisms of the *SCD* gene that are dependent on tissue type have also been confirmed in porcine (Doran et al., 2006) and cattle (Hiller et al., 2011) muscles and adipose tissues.

5 Conclusions

In summary, our research showed that diet supplementation with fresh red clover and grass significantly modified expression of *LPL*, *ACACA* and *SCD* genes in fat and liver tissues, which can be indirectly related to fatty acid concentration in animal products. Furthermore, tissue-specific regulations of *SCD* and *ACACA* expression were identified and the opposite effect of diet additions was observed depending on tissue type. The ability of nutrigenomic regulation of the analyzed gene transcription confirmed that these genes play a critical role in regulation of lipid metabolism processes in sheep and could be associated with fatty acid profiles in milk and meat.

Data availability. The original data of the paper are available upon request from the corresponding author.

The Supplement related to this article is available online at https://doi.org/10.5194/aab-60-243-2017-supplement.

Competing interests. The authors declare that they have no conflict of interest.

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