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Carotenoid intake and *SCD* genotype exert complementary effects over fat content and fatty acid composition in Duroc pigs[1]

E. Henriquez-Rodriguez,* R. N. Pena,* A. R. Seradj,* L. Fraile,* P. Christou,†‡ M. Tor,* and J. Estany*[2]

*Departament de Ciència Animal and †Departament de Producció Vegetal i Ciència Forestal, Universitat de Lleida-Agrotecnio Centre, Lleida, Catalonia, Spain; and ‡Institució Catalana de Recerca i Estudis Avançats, Barcelona, Catalonia, Spain

ABSTRACT: Nutritional and genetic strategies are needed to enhance intramuscular fat (IMF) and MUFA content without altering carcass leanness. Dietary vitamin A restriction has been suggested to specifically promote IMF, whereas a polymorphism of the *stearoyl-CoA desaturase* (*SCD*) gene has shown to specifically increase MUFA. The purpose of this study was to investigate the combined effects of provitamin A (PVA) carotenoid intake and *SCD* genotype (*AY487830:g.2228T>C*) on hepatic retinoid content and on the liver, muscle (LM and gluteus medius [GM]), and subcutaneous fat (SF) content and fatty acid composition. Following a split-plot design, 32 castrated Duroc pigs, half of each of the 2 homozygous *SCD* genotypes (CC and TT), were subjected from 165 to 195 d of age to 2 finishing diets differing in the PVA carotenoid content (an enriched-carotene diet [C+] and a control diet [C-]). Both diets were identical except for the corn line used in the feed. The C+ was formulated with 20% of a carotenoid-fortified corn (M37W-Ph3) whereas the C- instead used 20% of its near isogenic M37W line, which did not contain PVA carotenoids. No vitamin A was added to the diets. The C- was estimated to provide, at most, 1,300 IU of vitamin A/kg and the C+ to supply an extra amount of at least 800 IU vitamin A/kg. Compared with the pigs fed the C-, pigs fed with C+ had 3-fold more retinoic acid ($P < 0.01$) and 4-fold more *SCD* gene expression in the liver ($P = 0.06$). The diet did not affect performance traits and backfat thickness, but pigs fed the C+ had less fat (4.0 vs. 5.0%; $P = 0.07$) and MUFA (18.3 vs. 22.5%; $P = 0.01$) in the liver, less IMF (5.4 vs. 8.3%; $P = 0.04$) in the GM, and more fat content (90.4 vs. 87.9%; $P = 0.09$) and MUFA (48.0 vs. 46.6%; $P = 0.04$) in SF. The TT genotype at the *SCD* gene increased MUFA ($P < 0.05$) in all tissues (21.4 vs. 19.5% in the liver, 55.0 vs. 53.1% in the LM, 53.9 vs. 51.7% in the GM, and 48.0 vs. 46.7% in SF for TT and CC genotypes, respectively). Liver fat and MUFA content nonlinearly declined with liver all-*trans* retinoic acid, indicating a saturation point at relatively low all-*trans* retinoic acid content. The results obtained provide evidence for a complementary role between dietary PVA and *SCD* genotype, in the sense that the TT pigs fed with a low-PVA diet are expected to show higher and more monounsaturated IMF without increasing total fat content.

Key words: growth, intramuscular fat, liver, oleic acid, pork, vitamin A

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INTRODUCTION

Intramuscular fat (**IMF**) and MUFA content play major roles in meat quality and human nutrition. The continued selection for lean content practiced in commercial pig lines has led to IMF below recommended levels for consumer satisfaction (Wood et al., 2008). Several genetic (Ros-Freixedes et al., 2012) and nutritional (Doran et al., 2006) strategies have been investigated to enhance IMF content without increasing subcutaneous fat (**SF**). Regarding genetics, molecular markers with a specific effect over MUFA could be very

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[2]Corresponding author: jestany@ca.udl.cat

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useful for selection. This is the case of a mutation in the promoter of the *stearoyl-CoA desaturase* (*SCD*) gene, which increases MUFA but not IMF and SF content (Estany et al., 2014). As for nutritional strategies, adjusting dietary vitamin A at critical stages for IMF adipocyte formation can specifically promote IMF (Wang et al., 2016). Previous experiments in cattle and pigs provided evidence that restricting vitamin A intake in finishing diets improves IMF (D'Souza et al., 2003; Pickworth et al., 2012) and MUFA (Ayuso et al., 2015a,b) with minimal impact on carcass composition. These effects can be partly explained because vitamin A, after being absorbed, is converted into retinoic acid, which coregulates key adipogenic genes such as *SCD* (Wang et al., 2016). Interestingly, the mutation described in Estany et al. (2014) is positioned in the core sequence of several putative retinoic acid response elements.

The β-carotene and other provitamin A (**PVA**) carotenoids are a source of dietary vitamin A. Different staple crops biofortified with vitamins are available (Farre et al., 2011), including high-carotenoid corn producing high levels of β-carotene (Zhu et al., 2008). Therefore, the objective of this study was to investigate the combined effect of PVA carotenoid intake and *SCD* genotype on IMF and MUFA. The relationship of hepatic retinoids with liver *SCD* expression and fat content and composition is also discussed.

MATERIALS AND METHODS

All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida (agreement CEEA 02-04/14) and performed in accordance with authorization 7704 issued by the Catalan Ministry of Agriculture, Livestock, Fisheries and Food, Lleida, Spain.

Pigs and Experimental Design

Thirty-two castrated Duroc pigs, half of which carried the CC genotype and half the TT genotype for the *AY487830:g.2228T>C* polymorphism at the *SCD* gene (Estany et al., 2014), were chosen for the experiment. All of them were from the same Duroc genetic line (Ros-Freixedes et al., 2012), which was completely closed in 1991 and since then has been selected for an index including BW, backfat thickness (**BT**), and IMF (Solanes et al., 2009). The pigs were sampled from litters produced by 23 dams and 7 sires that were born within the range of 4 d. The piglets were castrated within the first week of age and, at 70 d (SD 1.3), moved to the *Centre d'Estudis Porcins* Pig Research Centre, Torrelameu, Lleida, Spain, where they were kept until slaughter in 8 pens of 4 pigs for a 2 × 2 split-plot arrangement. The 2 treatments consisted of 2 finishing diets differing in the PVA carotenoid content and the 2 *SCD* homozygotes. Therefore, each diet was given to 4 alternate pens, each one housing 2 CC and 2 TT piglets of similar weight. Sires were equally represented by diet, with at least 4 sires having offspring of each genotype.

Diets

The pigs were subjected to a 4-phase feeding program during the experiment. In all phases, the pigs had ad libitum access to cereal-based commercial diets, but only in the fourth phase, which was given during the finishing period from 165 to 195 d of age, the diets were formulated to specifically contain different amount of PVA carotenoids. In the first 3 phases (from 70 to 110 d, from 110 to 140 d, and from 140 to 165 d), 4 pens were fed a regular-protein diet (17, 16, and 15% CP, respectively) and 4 pens were fed a low-protein diet (15, 14, and 13% CP, respectively). Diets in the first phase were supplemented with 8,000 IU vitamin A per kilogram of feed, and diets in the second and third phase were supplemented with 6,500 IU per kilogram of feed. A full description of the diets used in these 3 first phases is given in Supplementary Table S1 (see the online version of the article at_http://journalofanimalscience.org). At the start of the fourth and last phase, 4 pens (2 from the regular-protein treatment and 2 from the low-protein treatment) were fed an enriched-carotene diet (**C+**) and 4 pens (2 from the regular-protein treatment and 2 from the low-protein treatment) were fed a control diet (**C-**). The 2 diets in the fourth phase were identical in terms of both ingredients and chemical composition (Table 1) except for the corn line used in the feed formulation. Therefore, the C+ was prepared using the M37W-Ph3 carotenoid-fortified corn (Zhu et al., 2008) and the C- was prepared using its near-isogenic M37W line, which contains only traces of carotenoids (primarily lutein and zeaxanthin). The nutrient composition of these 2 corn genetic types is reported in Supplementary Table S2 (see the online version of the article at http://journalofanimalscience.org). No vitamin–mineral premix, including vitamin A, was added in the formulation of the feed for the C+ and C-. The C- was estimated to provide, at most, 1,300 IU of vitamin A/kg and the C+ to supply an extra amount of at least 800 IU vitamin A/kg. These feeds were manufactured in 1 batch at the beginning of the fourth phase. Nutrient and fatty acid analyses were performed in duplicate as described in Cánovas et al. (2009). Feed protein was hydrolyzed by incubating a sample of 500 mg in 5 mL 6 *N* HCl for 12 h at 110°C. An aliquot of 25 μL of hydrolysate was evaporated under nitrogen steam and rediluted in 500 μL of water:acetonitrile (20:80, vol/vol). Quantitation of lysine was performed by using an ultra-performance liquid

chromatograph coupled to a triple quadrupole mass spectrometer system. Carotenoids in whole corn were extracted using the method of Folch et al. (1957) and analyzed as indicated below for liver retinoids.

Performance Traits and Sample Collection

Weekly records of BW were taken throughout the experiment. The average feed intake per pen was also calculated on a weekly basis. At the end of the finishing period, the pigs were humanely euthanized and carcass BT at 6 cm off the midline between the third and fourth last ribs was ultrasonically estimated (Renco Corp., Minneapolis, MN). Then, a representative sample of the liver, SF, and muscles gluteus medius (**GM**) and LM were taken and stored at −80°C until required.

Analysis of Vitamin A Metabolites in the Liver

Liver samples and standards were stored in the dark and handled under red light. Liver tissue samples where lyophilized and ground to powder in liquid nitrogen to avoid thermal degradation. An aliquot of 100 mg of freeze-dried tissue was weighed in a 15-mL Falcon tube (Corning Life Science, Tewksbury, MA). Then, 200 ng of all-*trans*-retinoic acid-D5 (Toronto Research Chemicals Inc., Toronto, ON, Canada) was spiked in 50 µL of acetonitrile:methanol:acetone (7:3:5, vol/vol/vol) containing 0.01% of butylated hydroxytoluene. At this point, 2 mL of acetonitrile:water (1:1, vol/vol) and 60 µL 4 *N* HCl were added to each sample, which was then vortexed for 30 min. Retinoids were extracted twice with 5 mL of hexane by vortexing for 5 min and centrifuging at 1,811 × *g* for 5 min at room temperature. Extracts were evaporated under a stream of nitrogen at 32°C and reconstituted in 1 mL of acetonitrile:methanol:acetone (7:3:5, vol/vol/vol) containing 0.01% butylated hydroxytoluene. Finally, extracts were filtered through a 0.20-µm polytetrafluoroethylene filter and transferred to an amber autosampler vial.

Quantitation of retinoids was performed using an Acquity UPLC system (Waters Corp., Milford, MA) equipped with an Acquity UPLC BEH C18 column (2.1 by 150 mm; 1.7 µm). Solvent A was 98:2 water:methanol with 0.1% formic acid; solvent B was 7:3 acetonitrile:methanol with 0.1% formic acid. The gradient included 4 steps. Initial conditions were 30% solvent A and 70% solvent B maintained for 1 min at 0.4 mL/min. Then, over 13 min, solvent B was increased from 30% to 100%, and from min 13 to 23, the flow was switched to 0.6 mL/min. From min 23 to 25, initial conditions were restored. Weak and strong washing solvents were 80:20 acetonitrile:water and isopropanol, respectively. The injection volume was 2.5 µL. Quantitation was performed by using a Multiple Reaction Monitoring method in a Waters XEVO TQD mass spectrometer (Micromass MS Technologies, Manchester, UK). The system was equipped with an atmospheric pressure chemical ionization source operated in positive ion mode. The parameters in the source were set as follows: 150°C source temperature, 450°C probe temperature, 150 L/h cone gas flow, and 0.25 mL/min collision gas flow. Retinoid transitions (Supplementary Table S3; see the online version of the article at http://journalofanimalscience.org) were determined by using commercial standards (all-*trans* retinol, all-*trans* retinal, keto-retinoic acid, all-*trans*-4-hidroxy retinoic acid, all-

Table 1. Ingredients and nutrient content of the diet fed during the finishing period (as-fed basis)

Item	g/kg
Ingredients	
Barley	571.5
Canola	90.3
Wheat shorts	52.6
Corn[1]	206.6
Blended animal:vegetal fat (3:5)	39.6
Monocalcium phosphate	9.4
Salt	8.9
Calcium carbonate	7.2
L-Lysine (50%)	5.4
DL-Methionine (99%)	0.7
L-Threonine	0.7
Nutrient content	
DM	875.1
CP[2]	121.5
Total lysine, %	0.7
GE, MJ/kg	16.0
Crude fat	61.2
Crude fiber	43.1
Ash	47.1
Fatty acids, %	
C16:0	20.4
Total SFA[3]	26.9
C18:1,*cis*-9	34.7
Total MUFA[4]	38.2
C18:2,*cis*-9,12	31.3
Total PUFA[5]	34.9

[1]The enriched-carotene diet used the fortified corn line M37W-Ph3 (7.0 ug/g DM of β-carotene) whereas the control diet used its near isogenic corn line M37W (nondetected β-carotene content). The enriched-carotene diet is estimated to provide 800 IU of vitamin A/kg more than the control diet.

[2]Amino acid content calculated according to De Blas et al. (2010): 0.68 % Lys, 0.38 % Ile, 0.26 % Met, 0.44 % Thr, 0.52 % Val, 0.49 % Met + Cys, and 0.12 % Trp.

[3]Fatty acids are expressed as percent of total fatty acids. Total SFA: C10:0 + C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0 + C24:0.

[4]Total MUFA: C14:1 *cis*-9 + C16:1 *cis*-9 + C17:1 *cis*-10 + C18:1 *trans*-9 + C18:1 *cis*-9 + C20:1 *cis*-11 + C22:1 *cis*-13.

[5]Total PUFA: C18:2 *trans*-9,12 + C18:2 *cis*-9,12 + C18:3 *cis*-6,9,12 + C18:3 *cis*-9,12,15 + C20:2 *cis*-11,14 + C20:3 *cis*-11,14,17 + C20:4 *cis*-5,8,11,14.

trans-retinoic acid, 9-*cis*-retinoic acid, 9,13-*cis*-retinoic acid, 13-*cis*-retinoic acid, all-*trans* retinoic-d5 acid, and all-*trans*-5-6-epoxy retinoic acid were purchased from Toronto Research Chemicals Inc., and retinyl palmitate was purchased from Sigma-Aldrich Química SL, Madrid, Spain). Cone voltage and collision energy were individually optimized. Calibration curves were constructed from the commercial compounds and diluted to a series of appropriate concentrations with acetonitrile:methanol:acetone (7:3:5, vol/vol/vol) containing 0.01% butylated hydroxytoluene. Data were processed using QuanLynx software (Waters, Milford, MA). For retinoid quantification, all-*trans* retinoic acid-d5 was used as an internal standard.

Fatty Acid Analysis

A representative aliquot from each pulverized freeze-dried sample was used for fat analysis. The IMF content and fatty acid composition of the liver, GM, LM, and SF were determined in duplicate by gas chromatography (Bosch et al., 2009). Fatty acid methyl esters were directly obtained by transesterification using a solution of 20% boron trifluoride in methanol (Rule, 1997). Methyl esters were determined by gas chromatography using a SP2330 capillary column (30 m by 0.25 mm; Supelco Inc., Bellefonte, PA) and a flame ionization detector with helium as carrier gas. Runs were made with a constant column-head pressure of 172 kPa. The oven temperature program increased from 150 to 225°C at 7°C/min, and injector and detector temperatures were both 250°C. The quantification was performed through area normalization after adding into each sample 1,2,3-tripentadecanoylglycerol as an internal standard. Intramuscular fat content was predicted as the sum of each individual fatty acid expressed as triglyceride equivalents (AOAC, 1997).

Analysis of Gene Expression

Total RNA was isolated from the liver of all pigs with TRI reagent (Sigma-Aldrich, St. Louis, MO) using the manufacturer's instructions. Two micrograms of RNA was digested with Turbo DNA-free DNase (Fisher Scientific, Madrid, Spain) and retrotranscribed into cDNA using RevertAid Premium Reverse Transcriptase (Fisher Scientific) using standard conditions with a mix of oligo(dT) and random primers at 1.25 μ*M* each. Reactions were incubated for 10 min at 25°C and 20 min at 37°C. Upon completion, cDNA were diluted 1:30 in H_2O and stored at −40°C. Quantitative PCR reactions for the *SCD* and 2 reference genes (*RPL32* and *B2M*) were set up in triplicate for each sample with primers and conditions as described by Estany et al. (2014).

Statistical Analysis

The effects of diet and *SCD* genotype were analyzed with a mixed model, which included the diet (C+ and C-), the genotype (TT and CC), and the diet × genotype interaction as fixed effects and the pen nested within diet as a random effect. Because feed intake was recorded on a pen basis, the model for feed intake and the feed conversion ratio included only the diet. The analyses were performed either with or without adding into the model the covariates age at slaughter (for BW, BT, fat content in the liver, and IMF) or fat content (for fatty acid composition traits). The potential effect of the diet given in earlier feeding phases was analyzed using the above model. No differences were observed between the regular-protein diet and the low-protein diet on target traits, and therefore, the previous diet was not included in the model. The relationship among retinoids was assessed using a multivariate REML correlation analysis, and that of retinoids with *SCD* gene expression and liver fat content and composition was assessed with a log–log regression analysis. Results were considered statistically significant at $P \leq 0.05$, whereas trending effects were discussed at $P \leq 0.10$. All the analyses were performed using the statistical package JMP 12 (SAS Inst. Inc., Cary, NC).

RESULTS

Upon absorption, PVA carotenoids are partially converted into vitamin A (retinol, retinal, retinoic acid, and intermediate compounds) mainly in the gut and liver. The liver, in addition, is the main site of vitamin A storage and metabolism. Therefore, we first assessed the impact of differential dietary carotenoid intake on the hepatic retinoid profile and then we assessed the combined effect of carotenoid intake and *SCD* genotype on fat content and composition. Results were used thereafter to investigate the relationship among liver retinoid content, *SCD* gene expression, and fat content and composition. Because the interaction between diet and the *SCD* genotype was only detected ($P < 0.10$) for a very low number of traits, means are separately presented for each factor and interactions are discussed only where relevant. In line with previously reported studies, means are presented adjusted for covariates, although results did not change when they were excluded from the analyses.

Effect of Diet and Genotype on Liver Retinoid Content

The diet, but not the genotype, influenced the retinoid content of the liver (Table 2). However, there was evidence of interaction between the diet and the *SCD* genotype for all-*trans* retinol ($P = 0.02$) and all-*trans*-5-6-epoxy retinoic acid ($P = 0.06$), for which the effect of the diet was evident only in the CC pigs. Therefore, for

Table 2. Concentration of retinoids in the liver by diet and *SCD* genotype

	Diet[2]		*SCD* genotype[3]		Diet × *SCD*
Retinoid,[1] µg/g DM	C-	C+	CC	TT	*P*-value
All-*trans* retinol	5.5 ± 1.2	8.7 ± 1.1	8.2 ± 1.2	6.0 ± 1.0	0.02
All-*trans* retinal	3.2 ± 0.4	4.2 ± 0.4	3.6 ± 0.5	3.7 ± 0.5	0.14
All-*trans* retinoic acid	0.2 ± 0.0[b]	0.5 ± 0.0[a]	0.3 ± 0.1	0.3 ± 0.0	0.26
13-*cis* retinoic acid	0.1 ± 0.0[b]	0.1 ± 0.0[a]	0.1 ± 0.0	0.1 ± 0.0	0.27
Epoxy retinoic acid (×10)	0.1 ± 0.0[b]	0.3 ± 0.0[a]	0.2 ± 0.0	0.2 ± 0.0	0.06
Retinol to retinal ratio	2.1 ± 0.3	2.0 ± 0.3	2.3 ± 0.2	1.8 ± 0.2	0.08
Retinoic acid to retinal ratio (×10)	0.3 ± 0.1[b]	0.6 ± 0.1[a]	0.4 ± 0.1	0.5 ± 0.1	0.41
Retinyl palmitate	782.8 ± 126.3	655.4 ± 122.1	680.7 ± 120.8	757.6 ± 108.9	0.16

[a,b]Within row and factor, means with different superscripts significantly differ ($P \leq 0.05$).

[1]Epoxy retinoic acid: all-*trans*-5-6-epoxy retinoic acid. All-*trans*-4-hidroxy retinoic acid, 9-*cis*-retinoic acid, 9,13-*cis*-retinoic acid, and keto-retinoic acid were analyzed but not detected.

[2]C+ = enriched-carotene diet: diet with 20% carotenoid-fortified corn M37W-Ph3; C- = control: diet with 20% of the near isogenic M37W line, which does not contain provitamin A carotenoids.

[3]CC and TT genotypes for the tag polymorphism *AY487830:g.2228T>C*.

all-*trans* retinol, there was a favorable effect of the C+ in the CC pigs (12.0 vs. 4.6 µg/g DM; $P < 0.01$) but not in the TT pigs (5.6 vs. 6.3 µg/g DM; $P = 0.77$). Feeding pigs the C+ increased all-*trans* retinoic acid (2.8-fold), 13-*cis* retinoic acid (1.8-fold), and all-*trans*-5-6-epoxy retinoic acid (2.2-fold) in the liver with respect to feeding pigs the C- ($P < 0.01$). This trend was also observed for all-*trans* retinol ($P = 0.10$) but not for all-*trans* retinal ($P = 0.16$) and retinyl palmitate content ($P = 0.50$). Other analyzed retinoids (all-*trans*-4-hidroxy retinoic acid, 9-*cis*-retinoic acid, 9,13-*cis*-retinoic acid, and all-*trans*-5-6-epoxy retinoic acid) were not detected in the liver. The correlations between liver retinoids were moderate to strong, ranging from 0.56 to 0.87, except for retinyl palmitate, the main storage form, which was not correlated with the rest of retinoids (Table 3).

Effect of Diet and Genotype on Fat Content and Composition

The diet and the *SCD* genotype had no effect on weight gain, feed intake, or the feed conversion ratio while pigs were raised on the experimental diets (Supplementary Table S4; see the online version of the article at http://journalofanimalscience.org). The difference observed between *SCD* genotypes in BW ($P = 0.03$) reflected the heavier BW of TT pigs at the start of the experimental feeding phase ($P = 0.05$). In general, the C+ decreased fat content in the liver and muscle (Table 4) but not in SF. Therefore, pigs fed the C+ had less fat in the liver (4.0 vs. 5.0%; $P = 0.07$), in the LM (4.2 vs. 4.8%; $P = 0.26$), and in the GM (5.4 vs. 8.3%; $P = 0.04$) than C--fed pigs. In contrast, the C+ not only did not affect BT ($P = 0.53$) but showed a trend to increase fat content in SF (90.4 vs. 87.9%; $P = 0.09$). The diet also had an impact on fatty acid composition, although with opposite effects in the liver (where MUFA decreased from 22.5% in C- to 18.3% in C+; $P = 0.01$) and SF (where MUFA increased from 46.6% in C- to 48.0% in C+; $P = 0.04$). Accordingly, the pigs fed the C+ had a lower MUFA to SFA ratio in the liver (0.53 vs. 0.65; $P = 0.01$). As expected, the *SCD* genotype did not alter fat content in any of the analyzed tissues but affected the MUFA to SFA ratio in all of them. Compared with CC pigs, the TT pigs showed a greater MUFA to SFA ratio in the liver, GM, LM, and SF due to an increment in MUFA at the cost of SFA (Table 4). The effects of the diet and the *SCD* genotype on each individual fatty acid together with the significance of their interactions are shown in Supplementary Tables S5, S6, S7, and S8 (see the online version of the article at http://journalofanimalscience.org) for the liver, LM, GM, and SF, respectively.

Relationship of Fat Content and Composition with Liver Retinoids

Liver fat content showed a negative correlation with liver retinoids, except for retinyl palmitate, where no association was detected. This general trend was most evident for liver all-*trans* retinoic acid ($P = 0.03$; Fig. 1). The plot of liver fat content against liver all-*trans* retinoic acid exhibited a nonlinear relationship, with greater expected responses at low levels of retinoids, indicating the existence of a saturation point at higher contents. Liver fatty acid composition also changed with liver retinoid content. In particular, MUFA in the liver was negatively correlated to all measured retinoids (see Fig. 2 for all-*trans* retinoic acid; $P < 0.01$) whereas the opposite was observed for PUFA. The only exception was for retinyl palmitate, which only showed a negative association with SFA ($P < 0.01$). These relationships were maintained after adjusting for fat content, thereby denoting that these

Table 3. Correlation among retinoids in the liver

Retinoid, μg/g DM	All-*trans* retinol	All-*trans* retinal	All-*trans* retinoic acid	13-*cis* retinoic acid	All-*trans*-5-6-epoxy retinoic acid	Retinyl palmitate
All-*trans* retinol	–	0.77*	0.69*	0.82*	0.86*	0.04
All-*trans* retinal		–	0.69*	0.61*	0.72*	−0.10
All-*trans* retinoic acid			–	0.59*	0.56*	−0.11
13-*cis* retinoic acid				–	0.87*	−0.22
All-*trans*-5-6-epoxy retinoic acid					–	−0.24
Retinyl palmitate						–

*$P \leq 0.05$.

compositional changes were due to a direct effect of retinoids on liver fatty acid composition rather than to an indirect effect resulting from correlated changes in fat content. The pattern of relationships of liver retinoids with IMF in the GM and LM was consistent with, although less strong than, that observed with liver fat content. In contrast, liver retinoids were not correlated with MUFA and the MUFA to SFA ratio in the GM, LM, and SF. However, a negative effect of the 3 retinoic acids on PUFA content in SF was found ($P < 0.05$).

Effect of Diet and Genotype on SCD *Gene Expression*

In the liver, the C+ induced a 4.5-fold increase of *SCD* gene expression compared with the C-, regardless of the *SCD* genotype (Fig. 3; $P = 0.06$). In line with this result, liver *SCD* gene expression increased with the 3 retinoic acids (Fig. 4 for all-*trans* retinoic acid; $P < 0.01$), with a similar trend for all-*trans* retinol ($P = 0.10$). No relationship between *SCD* gene expression and all-*trans* retinal ($P = 0.18$) and retinyl palmitate ($P = 0.17$) was detected. The higher expression of liver *SCD* was associated with lower ($P = 0.01$) MUFA and higher ($P = 0.01$) PUFA (Fig. 5) but not to changes in total liver fat and SFA. Regarding the other tissues, liver *SCD* expression was positively correlated to IMF in the GM ($P = 0.01$).

DISCUSSION

Vitamin A is crucial for many physiological functions, daily BW gain, and immune status. Because pigs are not very efficient at converting β-carotene to vitamin A, with only around one-third of the conversion efficiency observed in rats or poultry (Mc Dowell, 2000), commercial diets are regularly supplemented with PVA compounds. The 2 most common types of feed supplementation are PVA carotenoids (mostly β-carotene) and preformed vitamin A (mainly retinyl esters). Carotenes are absorbed and converted into all-*trans* retinoic acid mainly in the gut and the liver, where it is further processed into other active compounds. Excess vitamin A is stored in the liver as retinyl palmitate, which represents around 50 to 80% of total retinol (Blomhoff and Blomhoff, 2006).

The carotenoid-rich diet (C+) increased the content of retinoids in the liver, in line with reported trials supplying different amounts of commercial vitamin A (Olivares et al., 2009c, 2011; Tous et al., 2014). In the body, vitamin A is found in 3 main circulating vitamers differing in their oxidation state (Bonet et al., 2003): the hydroxyl form (retinol), the aldehyde form (retinal), and the carboxylic acid form (retinoic acid). Our results indicate that all 3 are well correlated and that retinol serves as a good predictor of the other 2. Reported values of liver retinol, the most abundant of the 3 and, as such, the most referenced retinoid, vary across experiments and species. The amount of liver retinol observed in our trial should be considered in the low range of reported values. However, the main retinoid molecule in the liver was retinyl palmitate (the storage form), which did not significantly differ between C--fed pigs and C+-fed pigs. Then, considering all retinoids, the observed total amount of vitamin A in terms of retinol equivalents was within the expected range (EFSA, 2008). Different reports suggest that PVA carotenoids and retinol have the same effect on liver retinol levels. Condron et al. (2014), in feedlot cattle, did not find any difference in liver retinol, retinyl palmitate, and retinoic acid between diets equivalently supplemented with synthetic β-carotene or retinyl palmitate. In laying hens, no differences in liver retinol content were found between diets supplemented with either high-carotenoid corn or retinol (Moreno et al., 2016).

Our experiment indicates that restricted PVA diets (such as C-) promote IMF deposition with no change in production traits, in line with previous findings in cattle (Siebert et al., 2006; Gorocica-Buenfil et al., 2007; Kruk et al., 2008; Pickworth et al., 2012) and pigs (D'Souza et al., 2003; Olivares et al., 2011; Ayuso et al., 2015a,b). Nonetheless, in pigs, the favorable effect of restricted vitamin A intake on IMF is less consistent across genetic types (Olivares et al., 2009b) and more variable with respect to the intensity and duration of the restriction period (Olivares et al., 2009c, 2011) and the target muscle (Ayuso et al., 2015a). Moreover, this effect was not replicated in all experiments (Olivares et al., 2009a; Tous et al., 2014). Several factors can explain such

Table 4. Least squares means (SE) for backfat thickness, intramuscular fat (IMF) content, and fatty acid composition in the liver, muscle, and subcutaneous fat by diet and *SCD* genotype

	Diet[1]		*SCD* genotype[2]		Diet × *SCD*
Trait[3]	C-	C+	CC	TT	*P*-value
No.	14	15	13	16	
Liver					
Fat, %	5.0 (0.3)	4.0 (0.3)	4.3 (0.3)	4.6 (0.3)	0.50
SFA, %	34.6 (0.2)	34.9 (0.2)	35.0 (0.2)	34.5 (0.2)	0.39
MUFA, %	22.5 (0.7)[a]	18.3 (0.7)[b]	19.5 (0.7)[b]	21.4 (0.6)[a]	0.94
PUFA, %	42.9 (0.7)[b]	46.8 (0.7)[a]	45.5 (0.6)	44.1 (0.6)	0.86
MUFA to SFA ratio	0.65 (0.02)[a]	0.53 (0.02)[b]	0.56 (0.02)[b]	0.62 (0.02)[a]	0.77
LM					
IMF, %	4.8 (0.4)	4.2 (0.3)	4.5 (0.3)	4.5 (0.3)	0.67
SFA, %	36.4 (0.4)	35.2 (0.4)	36.7 (0.4)[a]	34.9 (0.4)[b]	0.71
MUFA, %	53.5 (0.6)	54.6 (0.5)	53.1 (0.5)[b]	55.0 (0.5)[a]	0.27
PUFA, %	10.0 (0.4)	10.3 (0.4)	10.2 (0.3)	10.1 (0.3)	0.11
MUFA to SFA ratio	1.48 (0.03)	1.56 (0.03)	1.45 (0.03)[b]	1.58 (0.03)[a]	0.59
Gluteus medius					
IMF, %	8.3 (0.8)[a]	5.4 (0.8)[b]	6.8 (0.8)	6.9 (0.7)	0.55
SFA, %	34.5 (0.3)	34.4 (0.3)	35.3 (0.4)[a]	33.6 (0.4)[b]	0.65
MUFA, %	52.4 (0.5)	53.2 (0.5)	51.7 (0.5)[b]	53.9 (0.5)[a]	0.51
PUFA, %	13.0 (0.7)	12.4 (0.6)	13.0 (0.6)	12.4 (0.5)	0.34
MUFA to SFA ratio	1.52 (0.02)	1.56 (0.02)	1.47 (0.02)[b]	1.61 (0.02)[a]	0.80
Subcutaneous fat					
Backfat thickness, mm	24.7 (1.1)	25.8 (1.1)	24.5 (1.0)	26.0 (0.9)	0.37
Fat, %	87.9 (0.9)	90.4 (0.9)	88.7 (0.7)	89.5 (0.7)	0.78
SFA, %	36.1 (0.4)	36.1 (0.3)	37.4 (0.6)[a]	34.9 (0.5)[b]	0.61
MUFA, %	46.6 (0.4)[b]	48.0 (0.4)[a]	46.7 (0.4)[b]	48.0 (0.4)[a]	0.96
PUFA, %	17.4 (0.5)	15.8 (0.4)	16.1 (0.5)	17.0 (0.5)	0.62
MUFA to SFA ratio	1.31 (0.02)	1.33 (0.02)	1.26 (0.03)[b]	1.38 (0.03)[a]	0.74

[a,b]Within row and factor, means with different superscripts significantly differ ($P \leq 0.05$).

[1]C+ = enriched-carotene diet: diet with 20% carotenoid-fortified corn M37W-Ph3; C- = control diet: diet with 20% of the near isogenic M37W line, which does not contain provitamin A carotenoids.

[2]CC and TT genotypes for the tag polymorphism *AY487830:g.2228T>C*.

[3]Fat and IMF are expressed on a wet weight basis. Fatty acids are expressed as a percent of total fatty acids. In muscles and subcutaneous fat: SFA: C10:0 + C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0; MUFA: C16:1*n*-9 + C17:1*n*-7 + C18:1*n*-7 + C18:1*n*-9 + C20:1*n*-9; and PUFA: C18:2*n*-6 + C18:3*n*-3 + C20:2*n*-6 + C20:4*n*-6. In the liver, SFA also included C13:0 + C15:0 + C22:0; MUFA also included C15:1*n*-5 + C20:1*n*-9 + C24:1*n*-9; and PUFA also included C20:3*n*-6 + C20:5*n*-3 + C22:6*n*-3.

heterogeneous responses. To start with, pre-experimental conditions determine the amount of vitamin A stored in the liver, which can become high (O'Byrne and Blaner, 2013) and can provide active retinol compounds to the body for a certain amount of time. Also, differences in the timing of fat development among genetic types, tissues, and muscles are additional contributing factors. For instance, with respect to C+, the effect of C- on fat accumulation was greater in the GM (1.5-fold) than in the LM (1.14-fold), intermediate in the liver (1.25-fold), and negligible in SF. Therefore, IMF and BT are distinctly affected by dietary restriction of carotenoids, probably due to the differential effect of retinoic acid on lipid accumulation with age. The development of adipocytes is essential for fat accretion, and active vitamin A vitamers modulate adipogenesis through acting as a ligand for retinoic acid receptors. Studies in pigs have shown that preadipocyte hyperplasia is an early process, with a fast decline after 40 d of age, at a time when hypertrophy begins, markedly increasing with age (Dunshea and D'Souza, 2003). However, differences in fat development across breeds can modulate this timing. Therefore, in Iberian pigs, Ayuso et al. (2015b) showed that the preadipocyte number in the LM increased when vitamin A supplements were not added to the feed from 2 mo of age. Subcutaneous fat develops and matures prior to IMF, and therefore, preadipocyte formation and hyperplasia happen earlier in SF than in IMF. This means that during growth, hypertrophy is the key driver for SF deposition (Nakajima et al., 2011) and hyperplasia is the key driver for IMF (Damon et al., 2006; Hausman et al., 2014). Because vitamin A is expected to promote hyperplasia in preadipocytes and reduce hypertrophy in mature adipocytes, its intake at critical periods for IMF formation

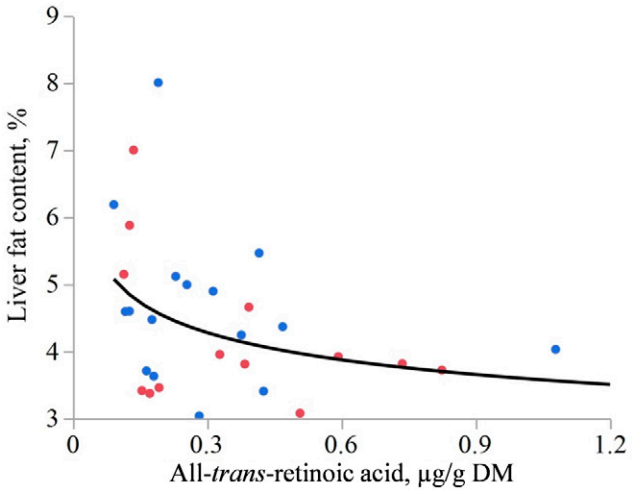

Figure 1. Relationship between fat content and all-*trans* retinoic acid [log (fat content) = 1.28 − 0.14 × log (all-*trans*-retinoic acid); $R^2 = 0.16$] in the liver of pigs with CC (●) and TT (●) genotypes at the *SCD* gene polymorphism *AY487830:g.2228T>C*.

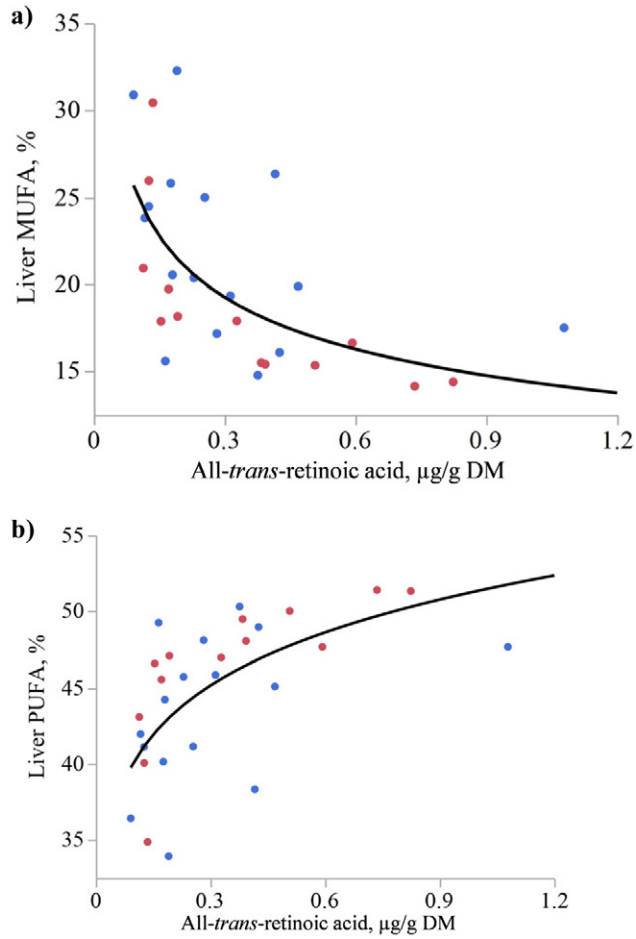

Figure 2. Relationship of (a) MUFA [log (MUFA) = 2.67 − 0.24 × log (all-*trans*-retinoic acid); $R^2 = 0.42$] and (b) PUFA [log (PUFA) = 3.94 + 0.11 × log (all-*trans*-retinoic acid); $R^2 = 0.37$] with all-*trans* retinoic acid in the liver of pigs with CC (●) and TT (●) genotypes at the *SCD* gene polymorphism *AY487830:g.2228T>C*.

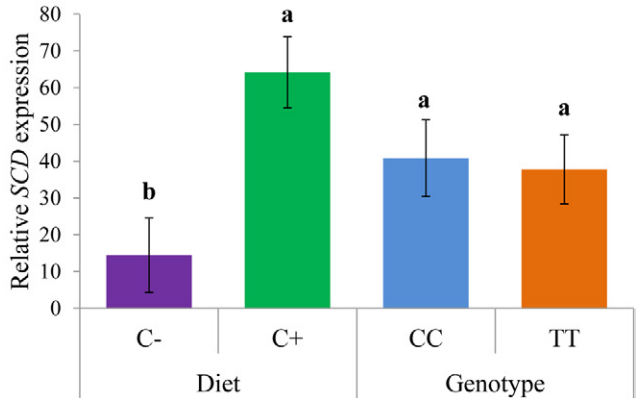

Figure 3. Least squares means for *SCD* gene expression in the liver by diet (control diet [C-]: diet with the near isogenic M37W line; enriched-carotene diet [C+]: diet with the carotenoid-fortified corn M37W-Ph3) and *SCD* genotype (CC and TT genotypes at the *SCD* gene polymorphism *AY487830:g.2228T>C*). a,bMeans with different letters within effect differ at $P \leq 0.10$.

and accretion can lead to enhance IMF content without increasing BT. It has been postulated that the differential effect of retinoic acid on progenitor cells and mature adipocytes is due to the stage-specific expression of related transcription factors (Wang et al., 2016). Altogether, our results are indicative that a carotenoid-restricted finishing diet, even for a short period, enables pigs to enhance IMF without substantial variation in BT. In accordance with the expected effects of vitamin A over fat deposition, it can be hypothesized that a greater response could have been obtained if restriction at finishing had been accompanied by vitamin A–enhanced starter or growing diets.

The pigs fed with C+ had higher levels of the 3 vitamers (retinol, retinal, and retinoic acid) in the liver than the control pigs and accumulated less fat in this tissue, confirming the inhibitory role of retinoids in fat deposition. The liver fat content decreases with all-*trans* retinoic acid in the liver, but the response was higher at low concentrations, suggesting a saturation point at relatively low values. Therefore, the relative high response of the diet on liver fat could be due to the low content of all-*trans* retinoic acid found in our trial as compared with other reported trials. Pigs fed the C- were provided with a feed containing the equivalent to 1,300 IU of vitamin A/kg, which roughly corresponds to the NRC recommended intake of vitamin A for growing pigs (NRC, 2012), and pigs fed the C+ had an extra daily intake of around 1,000 IU of vitamin A. These values of vitamin A intake allow us to postulate that a relatively small but continuous amount of carotenoid intake over the recommended level would suffice to induce changes in fat metabolism and beneficial effects in the prevention and treatment of fat liver–related diseases.

The effect of the diet showed an opposite pattern on the fatty acid composition of the liver and SF, with C+ decreasing MUFA in the liver but increasing it in SF. Carotenoids also increased liver *SCD* expression by 4.5-

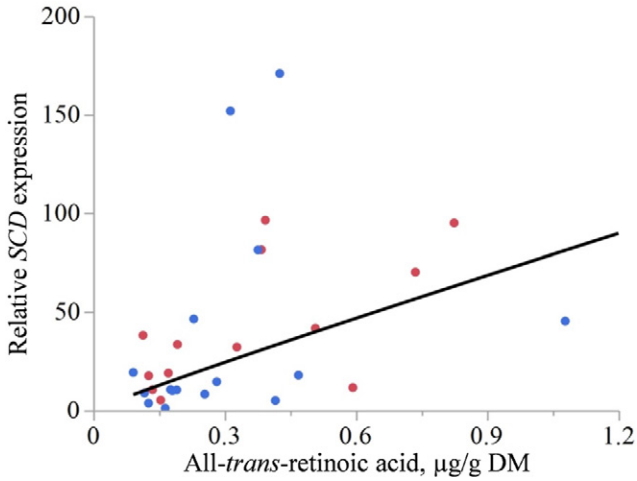

Figure 4. Relationship between *SCD* expression and all-*trans* retinoic acid [log (relative *SCD* expression) = 4.32 + 0.94 × log (all-*trans*-retinoic acid); R^2 = 0.26] in the liver of pigs with CC (●) and TT (●) genotypes at the *SCD* gene polymorphism *AY487830:g.2228T>C*.

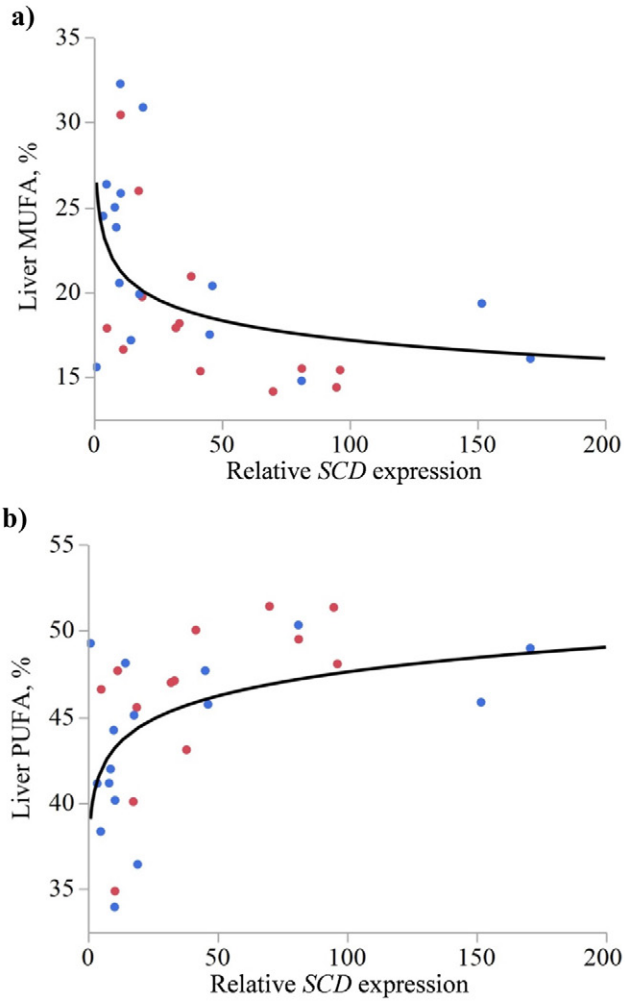

Figure 5. Relationship of (a) MUFA [log (MUFA) = 3.28 − 0.09 × log (relative *SCD* expression); R^2 = 0.22] and (b) PUFA [log (PUFA) = 3.67 + 0.04 × log (relative *SCD* expression); R^2 = 0.21] with *SCD* gene expression in the liver of pigs with CC (●) and TT (●) genotypes at the *SCD* gene polymorphism *AY487830:g.2228T>C*.

fold, in line with expectations (Ntambi and Miyazaki, 2004). This enzyme is directly involved in the biosynthesis of MUFA from SFA. However, in the liver, there is a clear inverse relationship between *SCD* expression levels and MUFA content. In the liver, the endogenously synthesized MUFA are the main substrate for the synthesis of hepatic triglycerides and cholesterol esters, which are released to the bloodstream in the form of very-low-density lipoprotein (**VLDL**; Hodson and Fielding, 2013). Therefore, in agreement with previous reports, enhanced *SCD* expression results in lower fat and MUFA content through the release of de novo fatty acids to circulating VLDL (Peter et al., 2011; Silbernagel et al., 2012), which indirectly affects liver PUFA content. Taken together, these results confirm the relationship between dietary PVA, liver *SCD* expression, and total fat and MUFA content in PUFA reported in lean humans and mice but not in obese individuals (Peter et al., 2011). Circulating MUFA are delivered in VLDL target tissues (muscle, heart, and adipose), increasing MUFA content in SF, the main tissue of fatty acid uptake. Previous studies in pigs have not found consistent results of dietary vitamin A on liver, SF, and muscle fatty acid profiles. In contrast with our results, Olivares et al. (2009a) found that dietary vitamin A increased SFA and decreased PUFA in the liver. The trends observed by Ayuso et al. (2015a), although not significant, were more in line with ours. Results in SF were also contradictory, with studies finding a negative effect of vitamin A on MUFA (Olivares et al., 2009a; Ayuso et al., 2015a) whereas others did not. In general, as here, little effects of dietary vitamin A were observed for muscle fatty acid profile, although some works described an increase in SFA (Ayuso et al., 2015a,b). In part, this likely could be because results in other experiments were not adjusted for fat content. Further research is needed to elucidate the role of vitamin A and its metabolites on fatty acid metabolism. However, in agreement with the diet effects, MUFA in the liver decreased (and PUFA increased) with all-*trans* retinoic content.

Consistent with earlier reports (Estany et al., 2014; Henriquez-Rodriguez et al., 2016), the *SCD* genotype affected fatty acid composition, with the TT genotype increasing MUFA and the MUFA to SFA ratio in all tissues, but not fat content. However, in contrast to diet, the major effect of the *SCD* genotype on fatty acid composition was in muscle and SF and not in the liver. In fact, the *SCD* genotype had no significant impact on hepatic *SCD* gene expression, in agreement with Estany et al. (2014), who found that the *SCD* genotype affected *SCD* gene expression in muscle and SF but not in the liver. This will confirm that the footprint of the *SCD* genotype is more important in IMF and SF, the sites where de novo fatty acid synthesis mostly occurs in pigs. Therefore, the positive effect of the TT genotype

on liver MUFA is difficult to interpret. But it is a fact that the effect of C+ on liver retinoids (all-*trans* retinol and all-*trans*-5-6-epoxy retinoic acid) was greater in the CC pigs, and some evidence indicated that the TT pigs could be less sensitive to the action of liver retinoids for *SCD* gene expression and MUFA ($P < 0.01$ for MUFA over all-*trans* retinal by *SCD* genotype). A more powerful experimental design is needed to confirm this result and to investigate the interaction between diet and *SCD* genotype, including retinoic acid and gene expression distribution throughout tissues. Duration, timing, and intensity of the restriction period as well as nutrient composition of the whole diets are also factors for further research. Results so far, however, provide evidence for a complementary role between dietary PVA and *SCD* genotype, in the sense that the TT pigs fed with a low-PVA diet are expected to show higher (because of the diet) and more monounsaturated (because of the *SCD* genotype) IMF without increasing total fat content.

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