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Differential effects of reduced protein diets on fatty acid composition and gene expression in muscle and subcutaneous adipose tissue of Alentejana purebred and Large White × Landrace × Pietrain crossbred pigs

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Abstract

The present study assessed the effect of pig genotype (fatty *v.* lean) and dietary protein and lysine (Lys) levels (normal *v.* reduced) on intramuscular fat (IMF) content, subcutaneous adipose tissue (SAT) deposition, fatty acid composition and mRNA levels of genes controlling lipid metabolism. The experiment was conducted on sixty intact male pigs (thirty Alentejana purebred and thirty Large White × Landrace × Pietrain crossbred), from 60 to 93 kg of live weight. Animals were divided into three groups fed with the following diets: control diet equilibrated for Lys (17·5 % crude protein (CP) and 0·7 % Lys), reduced protein diet (RPD) equilibrated for Lys (13·2 % CP and 0·6 % Lys) and RPD not equilibrated for Lys (13·1 % CP and 0·4 % Lys). It was shown that the RPD increased fat deposition in the longissimus lumborum muscle in the lean but not in the fatty pig genotype. It is strongly suggested that the effect of RPD on the longissimus lumborum muscle of crossbred pigs is mediated via Lys restriction. The increase in IMF content under the RPD was accompanied by increased stearoyl-CoA desaturase (*SCD*) and *PPARG* mRNA levels. RPD did not alter backfat thickness, but increased the total fatty acid content in both lean and fatty pig genotype. The higher amount of SAT in fatty pigs, when compared with the lean ones, was associated with the higher expression levels of *ACACA*, *CEBPA*, *FASN* and *SCD* genes. Taken together, the data indicate that the mechanisms regulating fat deposition in pigs are genotype and tissue specific, and are associated with the expression regulation of the key lipogenic genes.

Key words: **Pigs: Reduced protein diet: Intramuscular fat: Fatty acid composition: Lipid metabolism**

Pork is one of the most consumed meats in the European Union, with 22 010 778 tons of carcass produced in 2010[1]. However, as the consequence of genetic selection towards reduced subcutaneous fat, particularly in the case of white European breeds (Large White and Landrace), the amount of intramuscular or marbling fat (IMF) in commercial crossbred pigs has also been dramatically reduced[2]. Conversely, some pig breeds, like Alentejana and Iberian, have typically large amounts of subcutaneous and IMF, which are very precociously deposited in the carcasses[3]. IMF is one of the key meat quality traits. The sensory properties of pork, such as juiciness, tenderness and overall acceptability, are negatively affected when IMF is reduced below 2 %[4]. It was proposed that acceptable pork eating quality requires a minimum IMF of 2·5 %[5]. However, according to Daszkiewicz *et al.*[6], about 84 % of the carcasses from commercial pig genotypes have a longissimus lumborum muscle fat content below the level required for acceptable eating quality. In contrast to beef, IMF in pork is usually not visible and, hence, an increase in IMF should not result in the rejection of the meat by consumers due to marbling[7]. In addition, it is well-known that fatty acid composition of IMF plays an important role in meat

Abbreviations: ACACA, acetyl-CoA carboxylase; AOAC, Association of Official Analytical Chemists; CEBPA, CCAAT/enhancer-binding protein alpha; CRAT, carnitine *O*-acetyltransferase; FABP4, fatty acid-binding protein 4; FAME, fatty acid methyl esters; FASN, fatty acid synthase; IMF, intramuscular fat; INRB, Instituto Nacional dos Recursos Biológicos; LPL, lipoprotein lipase; P_2, last rib position; PPARA, PPAR alpha; PPARG, PPAR gamma; RPD, reduced protein diets; SAT, subcutaneous adipose tissue; SCD, stearoyl-CoA desaturase; SREBP1, sterol regulatory element-binding protein 1.

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quality, and therefore an appropriate proportion of SFA, MUFA and PUFA should be maintained in order to assure superior eating quality and nutritional value[8]. Therefore, production of pork with high amounts of IMF and a balanced fatty acid composition, without an increase in subcutaneous fat (improved fat partitioning), is highly desirable for the pig industry and consumers.

In pigs, the use of reduced protein diets (RPD)[9] or low lysine levels[10] has been proved to be the most successful nutritional strategy to enhance fat accumulation in muscle without a significant effect on subcutaneous adipose tissue (SAT). Although the principle of these strategies is to restrict muscle development, the mechanisms involved in the increasing of IMF content remain unknown[11]. One of the possible explanations might be the tissue-specific stimulation of expression of lipogenic enzymes under RPD, which, in turn, could lead to the increase of *de novo* fatty acid synthesis. One of the key lipogenic enzymes is stearoyl-CoA desaturase (SCD), which catalyses the rate-limiting step of MUFA biosynthesis. Da Costa *et al.*[12] showed that an RPD with a low lysine level increased SCD transcriptional rate in pig muscles. In line with this, Doran *et al.*[9] demonstrated that this increase in the transcriptional rate is followed by an increase in SCD protein expression and activity in muscles, but not in SAT from a commercial lean pig genotype (Duroc × Large White × Landrace). However, it remains unknown whether a combined reduction of dietary protein and lysine levels is required to increase IMF, and whether responses of fatty pig genotypes to RPD are similar to those of lean pig genotypes.

In addition to SCD, there are a number of other key enzymes and transcription factors involved in lipid metabolism. These factors determine the rates of *de novo* fatty acid biosynthesis, fat uptake from blood, transport of fatty acids in adipocytes and lipid degradation. Acetyl-CoA carboxylase (ACACA)[13] and fatty acid synthase (FASN)[14] are the key lipogenic enzymes controlling the rates of SFA biosynthesis. Lipoprotein lipase (LPL) is the rate-limiting enzyme for the conversion of chylomicrons and VLDL into chylomicron remnants and LDL in tissues. Therefore, LPL controls TAG partitioning between adipose tissue and muscle, thereby increasing fattening or providing energy in the form of fatty acids for muscle growth[15]. Furthermore, fatty acid-binding protein 4 (FABP4) is responsible for fatty acid transport in adipocytes[11]. Moreover, carnitine *O*-acetyltransferase (CRAT) is the rate-limiting enzyme of lipid catabolism, transporting fatty acid esters from cytosol to mitochondria for β-oxidation[16], whereas PPAR alpha (PPARA) is a major inducer of fatty acid oxidation[17]. It is also known that the transcription factors, sterol regulatory element-binding protein 1 (SREBP1), CCAAT/enhancer-binding protein alpha (CEBPA) and PPAR gamma (PPARG), are involved in the control of lipid metabolism in adipose tissue via regulation of expression of key enzymes and proteins controlling adipogenesis and lipogenesis[18–20]. The effects of dietary protein and lysine levels on the expression of genes encoding for lipid-metabolising enzymes are largely unknown.

To summarise, the genotype- and tissue-specific effects of RPD on fat partitioning and fatty acid composition in pigs, the interaction between dietary protein and lysine levels and the role of lipogenic enzymes and nuclear transcription factors in regulation of these effects remain to be elucidated. Therefore, in the present paper, we tested the following hypothesis: (1) the effect of RPD on fat partitioning between the muscle and subcutaneous depots is genotype specific; (2) the effect of RPD on fat partitioning is realised via the restriction in dietary lysine level; (3) the tissue-specific effect of RPD is mediated via the expression of key genes controlling lipid metabolism. To answer to the earlier questions, two distinct pig genotypes were chosen for the present study, the fatty Alentejana purebred and a lean commercial crossbred.

Materials and methods

Animals and diets

The present trial was conducted at the facilities of L-INIA (Instituto Nacional dos Recursos Biológicos (INRB)), and all the experimental procedures involving animals were reviewed by the Ethics Commission of the Centro de Investigação Interdisciplinar em Saridade Animal/Faculdade de Medicina Veterinária (CIISA/FMV) and approved by the Animal Care Committee of the National Veterinary Authority (Direcção-Geral de Veterinária) following the appropriate European Union guidelines (Directive 86/609/EEC). A total of thirty Alentejana purebred and thirty commercial crossbred (50 % Large White, 25 % Landrace and 25 % Pietrain) entire male pigs with an average initial body weight of 59·9 (SD 1·97) kg were used. Animals were fed a standard concentrate diet from weaning until the beginning of the experiment. Thereafter, animals from each breed were randomly assigned to one of the three diets in a 2 × 3 factorial arrangement (two breeds and three diets). The experimental diets were isoenergetically formulated (13·5 MJ metabolisable energy/kg calculated according to the NRC (1998)) and differed in crude protein and lysine contents as follows: 17·5 % of crude protein and 0·7 % of lysine (control diet); 13·2 % of crude protein and 0·6 % of lysine (RPD equilibrated for lysine, RPDL); and 13·1 % of crude protein and 0·4 % of lysine (RPD not equilibrated for lysine, RPD). L-Lysine was added to the RPDL diet to equilibrate the level of this amino acid with the control diet. The ingredients, chemical composition and fatty acid profile of the experimental diets are shown in Table 1. The animals were housed in two pens of four pigs each and one pen of two pigs per treatment (*n* 10). During the experiment, the animals were fed individually twice a day and had access to water *ad libitum*. Feed offered and refusals were recorded daily in order to calculate feed intake. Individual feed intake was recorded daily by refusal weighing. Pigs were weighed weekly, just before feeding, throughout the experiment.

Slaughter and sampling

Feed was removed 17–19 h before the slaughter of the animals. Pigs were slaughtered at an average live body weight of 93·4

(SD 2·42) kg, with no significant differences ($P>0·05$) among animal groups, at the L-INIA Experimental Abattoir (INRB). Immediately after electrical stunning and exsanguination, samples of the longissimus lumborum muscle and SAT for gene expression analysis were collected from the right side of the carcass at the 1st lumbar vertebra level, rinsed with sterile RNAse-free cold saline solution, cut into small pieces (thickness of about 0·3 cm), stabilised in RNA Later solution (Qiagen) and stored at −80°C until analysis. For the determination of IMF and fatty acid composition, longissimus lumborum muscle and SAT samples were collected after slaughter from the right side of the carcass between the L1 and L5 ribs. Muscle was collected and trimmed of visible connective and adipose tissues before blending in a food processor. The samples of muscle and SAT were vacuum packed and stored at −20°C until analysis. Backfat thickness was measured in the left carcass side at shoulder, P_2 (last rib position), last lumbar vertebra and second sacral vertebral locations.

Table 1. Ingredients, chemical and fatty acid compositions of the experimental diets

Diets...	Control	RPDL	RPD
Ingredients (%)			
Barley	40·0	50·0	50·0
Wheat	26·1	29·5	29·0
Soyabean meal	24·8	11·7	11·8
Maize	5·0	5·0	5·0
Soyabean oil	1·6	1·5	1·5
Calcium carbonate	1·1	1·1	1·1
Pigs vitatec	0·4	0·4	0·4
Salt	0·4	0·4	0·4
Di-calcium phosphate	0·2	0·4	0·4
L-Lys	0·0	0·2	0·0
Tecaphos 500 g	0·1	0·1	0·1
Ultracid V Dry EU	0·1	0·1	0·1
Grain Tec TS	0·1	0·1	0·1
Unilike Plus Dry	0·05	0·05	0·05
Oxi-Nil Dry Premix	0·003	0·003	0·003
Chemical composition (% diet)			
DM	89·1	88·9	89·0
Crude protein	17·5	13·2	13·1
Starch	47·2	54·9	55·2
Crude fat	3·1	2·9	2·9
Crude fibre	4·9	4·1	4·0
Ash	4·4	3·9	4·0
L-Lys	0·7	0·6	0·4
Ca	0·82	0·78	0·84
P	0·37	0·37	0·37
ME (MJ ME/kg)	13·3	13·6	13·5
Lys/ME	0·053	0·044	0·030
Fatty acid composition (% total fatty acids)			
14 : 0	0·1	0·1	0·2
16 : 0	17·3	18·7	19·6
16 : 1*c*9	0·2	0·2	0·2
18 : 0	2·6	2·6	2·6
18 : 1*c*9	19·0	18·9	19·3
18 : 1*c*11	1·5	1·5	1·6
18 : 2*n*-6	52·5	51·4	50·2
18 : 3*n*-3	4·9	4·6	4·3
20 : 0	0·3	0·3	0·3
20 : 1*c*11	0·4	0·5	0·5

Control, normal protein diet equilibrated for lysine; RPDL, reduced protein diet equilibrated for lysine level; RPD, reduced protein diet not equilibrated for lysine level; ME, metabolisable energy.

Feed analysis

Feed samples, collected four times during the trial (in the beginning and on a 3-week regular period), were analysed for DM by drying a sample at 100°C to a constant weight. N content was determined by Kjeldahl[21] and crude protein was calculated as 6·25 × N. Crude fibre was determined by the procedure described by the Association of Official Analytical Chemists (AOAC)[21]. The samples were extracted with petroleum diethyl ether, using an automatic Soxhlet extractor (Gerhardt Analytical Systems), to determine crude fat. Determination of ash and starch contents was carried out according to the procedures described by the AOAC[21] and Clegg[22], respectively. Gross energy in the feed was determined by adiabatic bomb calorimetry (Parr 1261, Parr Instrument Company). Fatty acid methyl esters (FAME) of the feed samples were analysed by one-step extraction and transesterification, using heptadecaenoic acid (17 : 0) as an internal standard[23]. Total amino acids were extracted from feed according to the method described by the AOAC[24]. The extract was analysed by HPLC (Agilent 1100, Agilent Technologies) to quantify amino acids in the feed, including lysine, according to the procedure reported by Henderson *et al.*[25].

Intramuscular fat and fatty acid composition

The longissimus lumborum muscle and SAT samples were lyophilised (−60°C and 2·0 hPa) to constant weight using a lyophilisator (Edwards High Vacuum International), kept dry at −20°C and analysed within 2 weeks. The total fat content of muscle samples (IMF) was determined using fresh samples by hydrolysis with 4 M-HCl followed by Soxhlet extraction for 6 h with petroleum ether[21]. For fatty acid analysis of longissimus lumborum muscle and SAT samples, FAME were extracted from the lyophilised samples (approximately 250 and 50 mg, respectively), according to the Folch *et al.*[26] method, using dichloromethane and methanol (2:1, v/v) instead of chloroform and methanol (2:1, v/v), as described by Carlson[27]. All the extraction solvents contained 0·01 % butylated hydroxytoluene as an antioxidant. Fatty acids were converted to methyl esters by a combined transesterification procedure with NaOH in anhydrous methanol (0·5 M), followed by HCl–methanol (1:1, v/v), at 50°C for 30 and 10 min, respectively, according to Raes *et al.*[28]. Quantification of FAME in muscle and SAT was performed using a gas chromatograph HP6890A (Hewlett-Packard), equipped with a flame ionisation detector (GC-FID) and a CP-Sil 88 capillary column (100 m × 0·25 mm inner diameter, 0·20 μm film thickness; Chrompack, Varian Inc.), using the conditions described in Alves & Bessa[29]. The quantification of total FAME was done using nonadecanoic acid (19 : 0) as the internal standard. Results for each fatty acid were expressed as a percentage of the sum of detected fatty acids (% total fatty acids).

RNA isolation and complementary DNA synthesis

Total RNA was isolated and purified from muscle and SAT using the Qiagen RNeasy fibrous tissue mini kit (Qiagen)

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and Qiagen RNeasy lipid tissue mini kit (Qiagen), respectively. Prior to RT-PCR, the total RNA samples were treated with DNAse I (Qiagen). All the procedures were performed in accordance with the manufacturer's protocols. RNA was quantified using a NanoDrop ND-2000c spectrophotometer (Nanodrop, Thermo Fisher Scientific). The A260/280 ratios were between 1·9 and 2·1. Ethidium bromide staining of 18S and 28S ribosomal bands was used to verify the sample integrity. Reverse transcription was performed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Briefly, each 20 μl RT reaction containing 1 μg of DNase-treated total RNA template, 50 nM random RT primer, 1× RT buffer, 0·25 mM of each deoxyribonucleotide triphosphate (dNTP), 3·33 U/μl multiscribe RT and 0·25 U/μl RNase inhibitor, was submitted to 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The complementary DNA solution obtained was divided into aliquots and stored at −20°C until further analysis.

Real-time quantitative PCR

Gene-specific intron-spanning primers were designed using Primer3 (http://frodo/wi.mit.edu/primer3) and Primer Express Software v. 2.0 (Applied Biosystems) based on *Sus scrofa* sequences (http://www.ncbi.nlm.nih.gov). Primers were synthesised commercially by NZYTech (Lisbon, Portugal). Sequence homology searches against the database of GenBank showed that these primers matched only with the sequence to which they were designed. To ensure optimal DNA polymerisation efficiency, the amplicon length ranged between 71 and 138 bp. Before performing the real-time quantitative PCR experiments, a conventional PCR was carried out for all genes investigated in order to test the primers and verify the amplified products. To confirm the identity of amplified fragments, PCR products were sequenced and homology searches were performed with Blast (http://www.ncbi.nlm.nih.gov/blast). In order to find the most stable endogenous control in SAT and longissimus lumborum muscle, five commonly used housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 60S ribosomal protein L27 (*RPL27*), ornithine decarboxylase antizyme 1 (*OAZ1*), ribosomal protein large P0 (*RPLP0*) and 40S ribosomal protein S29 (*RPS29*) were used to normalise the results of target genes. Expression level stability of housekeeping genes was analysed using the geNorm (http://medgen.ugert.be/~jrdesomp/genorm)[30] and NormFinder (http://www.mdl.dk/publicationsnormfinder.htm)[31] software packages as described in their manuals. The *RPLP0* and *RPS29* genes were selected as the most stable pair of internal controls for normalisation. The sequence of primers (including annealing temperatures), GenBank accession numbers, PCR efficiency, regression coefficients and span exons for PCR products are provided in Table 2. PCR efficiency was calculated for each amplicon using StepOnePlus PCR System software (Applied Biosystems), by amplifying 5-fold serial dilutions of pooled complementary DNA and run in triplicate. All primer sets exhibited an efficiency that ranged between 90 and 110 %, and correlation coefficients were higher than 0·99. real time quantitative PCR were carried out using MicroAmp Optical

Table 2. Characterisation of the selected genes used in the real-time quantitative PCR assay

Gene symbol	Full gene name	GenBank accession no.	PCR efficiency SAT/LL muscle	Regression coefficient (r^2) SAT/LL muscle	Forward primer	Reverse primer	Product size (bp)	Spanned coding exons	Annealing temperature (°C)
Key lipid metabolism genes									
ACACA	Acetyl-CoA carboxylase, alpha	NM_001114269	97·1/90·5	0·997/0·996	ggccatcaaggacttcaacc	acgatgtaagcgccgaactt	120	46–47	58·0/58·5
CEBPA	CCAAT/enhancer-binding protein (C/EBP) alpha	XM_003127015	91·78/100·0	0·994/0·990	ggccagcacacacacattaga	cccccaaagaagagaaccaag	71	1	58·2/58·7
CRAT	Carnitine *O*-acetyltransferase	NM_001113047	98·86/92·96	0·996/0·999	ggcccaccgagcctacac	atggcgatggcgtaggag	138	12–13	60·0/58·2
FABP4	Fatty acid-binding protein 4	NM_001002817	98·0/91·77	1·0/0·996	gggccaggaatttgatgaag	ctttccatcccacttctgcac	103	2–3	58·0/58·2
FASN	Fatty acid synthase	NM_001099930	92·45/91·07	0·999/0·999	acaccttcgtgctggcctac	atgtcggtgaactgctgcac	112	40–41	58·8/58·3
LPL	Lipoprotein lipase	NM_214286	93·15/91·15	0·999/0·997	atctgcgggatacaccaagc	ccaaggctgtatcccaggag	110	3–4	58·6/58·2
PPARA	Peroxisome proliferator-activated receptor alpha	NM_001044526	97·92/100·16	0·995/0·998	tttccctctttgtggctgct	ggggtggttggtctgcaag	128	5–6	58·5/59·9
PPARG	Peroxisome proliferator-activated receptor gamma	NM_214379	97·88/98·5	0·999/0·993	gagggcgatcttgacaggaa	gccacctctttgctctgctc	124	6–7	58·8/58·8
SCD	Stearoyl-CoA desaturase	NM_213781	94·86/93·16	0·997/0·998	agccgagaagctggtgatgt	gaagaaaggtggcgacgaac	140	5–6	58·5/58·4
SREBP1	Sterol regulatory element-binding protein 1	NM_214157	102·0/96·43	0·999/0·999	gtgctggcggaggtctatgt	aggaagaagcgggtcagaaag	96	11–12	58·8/58·6
Housekeeping genes									
RPLP0	Ribosomal phosphoprotein large, P0 subunit	NM_001098598	95·36/97·14	0·998/0·999	tccaggctttaggcatcacc	ggctcccactttgtctccag	95	4–5	58·7/58·3
RSP29	Ribosomal protein S29	NM_001001633	96·65/97·57	1·0/1·0	ggtcagggttctcgctcttg	cactggcggcacatattgag	120	1–2	58·5/58·7

SAT, subcutaneous adipose tissue; LL, longissimus lumborum.

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ninety-six-well plates (Applied Biosystems) in a StepOnePlus thermocycler (Applied Biosystems) under standard cycling conditions. The 12·5 μl PCR mixtures contained 6·25 μl of 2 × Power SYBR Green PCR Master Mix (Applied Biosystems), 160 nM of forward and reverse primers and 2 μl of diluted complementary DNA as template. No transcription and no template samples were used as controls. The primer specificity and the formation of primer–dimers were confirmed by melt curve analysis and agarose gel electrophoresis. All analyses were performed in duplicate, and the relative amounts for each target gene was calculated using the geometric mean of *RPLP0* and *RPS29* as a normaliser. The relative expression levels were calculated as a variation of the Livak method[32], corrected for variation in amplification efficiency, as described by Fleige *et al.*[33].

Statistical analysis

For IMF content and fatty acid composition, all experimental groups were considered. As the RPDL had no significant effect on IMF and SAT deposition, relative to the control diet, gene expression analysis was performed only on four experimental groups (Alentejano and crossbred pigs fed with the control and RPD diets). All data were checked for normal distribution and variance homogeneity. As variance heterogeneity was detected for most of the variables, data were analysed using Proc MIXED of the SAS software package[34] (version 9.2; SAS Institute), with a model including the breed, diet and their respective interaction as fixed effects and the repeated statement considering the group option to accommodate the variance heterogeneity. The level of significance was set at $P<0·05$.

The need for covariate adjustment was explored using age, live and slaughter weights, IMF and P_2 as covariates, but only IMF and P_2 revealed to be significant for several variables. Thus, IMF and P_2 were retained as covariates for some muscle and SAT variables, respectively. For each variable, where the use of a covariate was justified, the structure of the covariate model was determined according to the procedures described by Milliken & Johnson[35] and ranged from a simple slope model to individual slopes for each diet × breed combinations. The adjusted variables and their covariance models are identified in the footnotes of the tables. As large differences in covariate ranges were intrinsically associated to each breed, the variable was adjusted and compared with the mean covariate value of each breed[35]. When significant effects were detected, least square means (LSMEANS) were determined using the LSMEANS option and compared using the probability difference procedure adjusted for multiple comparisons using the Tukey–Kramer method.

Pearson correlation matrices were computed using the PROC CORR of SAS. When needed, adjusted variables to the common mean IMF in muscle and the common mean P_2 in SAT were used to compute Pearson correlations.

Results

The results of the present trial regarding pigs' performance, carcass traits and sensory quality of meat were obtained (MS Madeira, P Costa, CM Alfaia, PA Lopes, RJB Bessa, JPC Lemos and JAM Prates, unpublished results). However, here we present and discuss the effects of RPD, with (RPDL) or without (RPD) equilibrated levels of lysine, on fatty acid content and composition of muscle and SAT from lean (commercial crossbred) and fatty (Alentejana purebred) pig genotypes. Furthermore, in order to elucidate the mechanisms underlying fat deposition in longissimus lumborum muscle and SAT obtained for the RPD in crossbred pigs, the expression level of genes encoding for key lipogenic enzymes and transcription factors involved in lipid metabolism was also assessed. As no significant effects ($P>0·05$) in IMF deposition were obtained for the RPDL, when compared with the control diet, the expression level of key genes involved in lipid metabolism were not investigated for the experimental groups fed this diet.

Intramuscular fat and fatty acid composition of muscle

Results of IMF, fatty acid composition, partial sums of fatty acids and related ratios in the longissimus lumborum muscle are presented in Table 3. In relation to IMF content, a significant interaction between breed and diet ($P=0·037$) was observed, with no dietary effect for Alentejano pigs, but with an increase of IMF by 40 % for the RPD in crossbred animals. In contrast, the RPDL did not increase ($P>0·05$) IMF, neither in Alentejano nor in crossbred pigs.

In all experimental groups, the predominant fatty acids in IMF were 18 : 1*c*9 (33–38 % of total FAME), 16 : 0 (23–26 %), 18 : 0 (12–14 %), 18 : 2*n*-6 (7–12 %) and 18 : 1*c*11 (5–6 %). It should be noted that 18 : 1 *trans* represents the sum of 18 : 1 *trans* 6 to *trans* 11. The term 'others' refers to unidentified minor fatty acids and the dimethylacetals 16 : 0, 18 : 0 and 18 : 1, which are derived from plasmalogens. The breed and diet interaction influenced only three fatty acids (12 : 0, 16 : 1*c*9 and 18 : 1*c*11). The breed affected fourteen of the nineteen fatty acids identified. The proportion of 16 : 0 ($P<0·001$), 18 : 0 ($P<0·001$), 18 : 1*c*9 ($P<0·001$) and 20 : 0 ($P = 0·003$) was highest in Alentejana purebred animals, when compared with the crossbred genotype. This is in contrast to the 14 : 0, 17 : 0, 18 : 2*n*-6, 18 : 3*n*-3, 20 : 3*n*-6 and 20 : 4*n*-6 fatty acids, which were highest in crossbred pigs. In addition, the dietary protein and lysine levels affected eight individual fatty acids in the longissimus lumborum muscle. The proportion of 16 : 0 was higher ($P=0·001$) in the pigs fed RPD, when compared with the animals fed control diet. Contrarily, 16 : 1*c*7 ($P=0·026$), 18 : 2*n*-6 ($P=0·010$), 18 : 3*n*-3 ($P=0·004$), 20 : 2*n*-6 ($P=0·015$) and 20 : 3*n*-6 ($P=0·002$) were lower in the RPD than in the control diet.

Regarding partial sums of fatty acids (Table 3), the observed patterns reflect the values described earlier for the major individual fatty acids of each group. Both the breed ($P<0·001$) and the diet influenced SFA ($P \leq 0·001$), PUFA ($P=0·009$) and *n*-6 PUFA ($P=0·010$). The proportion of SFA was higher in the RPD relative to the control diet, while the proportions of PUFA and *n*-6 PUFA and the PUFA:SFA ratio were lower in the RPD.

Fatty acid content and composition of subcutaneous adipose tissue

Table 4 shows backfat thickness at P_2 site, total fatty acids, fatty acid composition and related indices for SAT. Regarding

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Table 3. Effect of the reduced protein diets equilibrated (RPDL) and not equilibrated (RPD) for lysine levels on intramuscular fat (IMF; % muscle), fatty acid composition (% total fatty acids), partial sums of fatty acids and related ratios in the longissimus lumborum muscle of Alentejana breed and crossbred pigs

(Mean values with their standard errors)

	Alentejana breed						Crossbred						Significance level		
	Control		RPDL		RPD		Control		RPDL		RPD				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Breed	Diet	Breed × diet
IMF	4·16	0·360	5·79	0·916	4·47	0·387	2·68	0·280	2·16	0·158	3·74	0·346	< 0·001	0·143	0·037
Fatty acid composition															
12 : 0	0·09[a,b]	0·009	0·07[a]	0·003	0·08[a,b]	0·005	0·08[a,b]	0·005	0·09[b]	0·002	0·08[a]	0·004	0·483	0·602	0·004
14 : 0*	1·31	0·047	1·40	0·043	1·45	0·042	1·52	0·041	1·51	0·072	1·46	0·053	0·011	0·661	0·125
16 : 0*	25·3	0·35	26·0	0·11	26·1	0·19	23·6	0·12	25·1	0·49	24·1	0·35	< 0·001	0·001†	0·305
16 : 1*c*7*	0·25	0·011	0·22	0·010	0·21	0·009	0·24	0·009	0·24	0·016	0·21	0·012	0·621	0·026‡	0·518
16 : 1*c*9*	2·76[a]	0·102	3·06[a,b]	0·093	3·14[a,b]	0·091	3·20[b]	0·089	3·43[b]	0·156	2·93[a,b]	0·115	0·031	0·060	0·004
17 : 0§	0·23	0·017	0·19	0·011	0·18	0·008	0·29	0·023	0·28	0·023	0·31	0·038	< 0·001	0·286	0·416
18 : 0	13·3	0·24	13·5	0·13	13·3	0·16	11·7	0·19	12·3	0·25	12·5	0·27	< 0·001	0·091	0·213
18 : 1*t*	0·13	0·009	0·15	0·007	0·15	0·006	0·16	0·012	0·15	0·009	0·15	0·004	0·236	0·895	0·421
18 : 1*c*9§	37·5	0·64	37·6	0·66	37·7	0·63	34·4	0·63	34·0	0·64	35·8	0·65	< 0·001	0·307	0·457
18 : 1*c*11*	5·21[a]	0·242	5·80[a,b]	0·221	5·79[a,b]	0·217	6·37[b]	0·213	5·69[a,b]	0·372	5·72[a,b]	0·273	0·127	0·980	0·019
18 : 2*n*-6*	8·04	0·447	7·00	0·408	6·94	0·400	10·6	0·39	9·07	0·686	9·37	0·404	< 0·001	0·010‖	0·857
18 : 3*n*-3*	0·37	0·018	0·34	0·016	0·33	0·016	0·44	0·016	0·38	0·028	0·37	0·020	0·003	0·004‡	0·480
20 : 0	0·17	0·008	0·16	0·007	0·16	0·006	0·14	0·013	0·13	0·006	0·14	0·007	0·003	0·577	0·804
20 : 1*c*11§	0·66	0·027	0·65	0·027	0·64	0·027	0·65	0·026	0·57	0·027	0·63	0·027	0·126	0·289	0·332
20 : 2*n*-6*	0·94	0·013	0·21	0·013	0·20	0·013	0·33	0·013	0·31	0·014	0·29	0·015	< 0·001	0·015‡	0·944
20 : 3*n*-6*	0·21	0·020	0·15	0·018	0·16	0·017	0·32	0·017	0·23	0·030	0·26	0·022	< 0·001	0·002‖	0·750
20 : 3*n*-3*	0·04	0·007	0·04	0·006	0·04	0·006	0·05	0·006	0·07	0·010	0·07	0·008	< 0·001	0·145	0·071
20 : 4*n*-6*	1·29	0·166	0·95	0·151	1·08	0·148	2·16	0·146	1·38	0·254	1·99	0·19	< 0·001	0·015¶	0·391
22 : 4*n*-6*	0·22	0·029	0·18	0·026	0·19	0·026	0·40	0·025	0·28	0·044	0·34	0·032	< 0·001	0·046¶	0·508
Others*	2·47	0·212	2·04	0·193	2·07	0·190	3·24	0·186	2·37	0·326	3·09	0·239	< 0·001	0·030¶	0·370
Fatty acid partial sums															
SFA*	40·3	0·45	41·4	0·41	41·3	0·40	37·3	0·39	39·8	0·69	38·8	0·51	< 0·001	0·001†	0·393
MUFA*	46·8	0·77	47·7	0·71	47·7	0·69	45·1	0·68	46·1	1·19	45·4	0·87	0·009	0·517	0·926
PUFA*	10·4	0·65	8·87	0·595	8·93	0·584	14·4	0·57	11·7	1·00	12·7	0·73	< 0·001	0·009‖	0·736
n-6 PUFA*	9·99	0·644	8·49	0·588	8·56	0·577	13·9	0·57	11·2	0·99	12·2	0·73	< 0·001	0·010¶	0·731
n-3 PUFA*	0·41	0·020	0·38	0·018	0·37	0·018	0·49	0·017	0·46	0·031	0·44	0·022	< 0·001	0·074	0·981
Fatty acid ratios															
PUFA:SFA*	0·26	0·019	0·21	0·017	0·22	0·017	0·39	0·017	0·29	0·029	0·33	0·021	< 0·001	0·003‖	0·423
n-6:*n*-3*	24·2	1·54	22·1	1·41	23·2	1·38	28·3	1·36	25·0	2·64	28·0	1·74	0·006	0·280	0·862

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
* Variable adjusted for breed × diet × IMF interaction.
† Control < RPDL, control < RPD, RPDL = RPD.
‡ Control = RPDL, control > RPD, RPDL = RPD.
§ Variable adjusted for IMF.
‖ Control > RPDL, control > RPD, RPDL = RPD.
¶ Control > RPDL, control = RPD, RPDL = RPD.

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Table 4. Effect of the reduced protein diets equilibrated (RPDL) and not equilibrated (RPD) for lysine levels on backfat thickness P_2 (last rib position, mm), total fatty acids (% fat), fatty acid composition (% total fatty acids), partial sums of fatty acids and related ratios in subcutaneous adipose tissue of Alentejana breed and crossbred pigs

(Mean values with their standard errors)

	Alentejana breed						Crossbred						Significance level		
	Control		RPDL		RPD		Control		RPDL		RPD				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Breed	Diet	Breed × diet
P_2 backfat thickness	28·0	1·33	32·0	2·02	29·8	1·33	14·7	0·97	14·8	1·14	15·8	1·74	< 0·001	0·318	0·378
Total fatty acids	55·3	0·97	56·7	0·68	59·0	0·78	52·4	1·68	52·7	1·31	54·5	1·25	< 0·001	0·049*	0·819
Fatty acid composition															
12 : 0	0·05	0·004	0·06	0·003	0·06	0·003	0·06	0·004	0·07	0·004	0·06	0·004	0·093	0·342	0·177
14 : 0	1·42^{a,b}	0·023	1·43^{a,b}	0·017	1·48^{b}	0·024	1·41^{a,b}	0·038	1·46^{a,b}	0·044	1·35^{a}	0·033	0·180	0·591	0·038
16 : 0†	25·5^{b,c}	0·29	25·9^{b,c}	0·28	26·4^{c}	0·26	24·1^{a}	0·26	24·9^{a,b}	0·26	23·9^{a}	0·26	< 0·001	0·146	0·002
16 : 1*c*7	0·22	0·006	0·20	0·009	0·19	0·011	0·30	0·010	0·28	0·009	0·28	0·009	< 0·001	0·008*	0·841
16 : 1*c*9	1·56	0·067	1·59	0·092	1·76	0·064	1·77	0·091	1·80	0·129	1·63	0·081	0·171	0·951	0·058
17 : 0‡	0·38	0·025	0·29	0·015	0·28	0·012	0·47	0·042	0·47	0·024	0·48	0·074	< 0·001	0·294	0·252
18 : 0§	15·8	0·46	16·0	0·47	15·8	0·46	14·8	0·46	14·8	0·457	15·2	0·458	0·061	0·672	0·885
18 : 1*t*‖	0·17	0·012	0·21	0·021	0·17	0·010	0·23	0·019	0·18	0·019	0·20	0·018	0·226	0·108	0·148
18 : 1*c*9	34·3	0·63	34·4	0·44	34·9	0·66	30·4	0·53	30·4	0·39	31·9	0·68	< 0·001	0·181	0·654
18 : 1*c*11†	8·08	0·423	7·81	0·404	7·27	0·382	7·45	0·385	7·20	0·383	8·00	0·385	0·602	0·808	0·141
18 : 2*n*-6†	9·81	0·233	9·08	0·226	8·87	0·193	15·3	0·452	14·6	0·30	13·4	0·624	< 0·001	0·005¶	0·434
18 : 3*n*-3†	0·80	0·019	0·72	0·019	0·70	0·019	1·05	0·032	0·96	0·021	0·88	0·045	< 0·001	< 0·001*	0·537
20 : 0	0·21	0·012	0·23	0·010	0·22	0·009	0·18	0·009	0·18	0·007	0·22	0·017	0·003	0·077	0·222
20 : 1*c*11	1·01^{b}	0·033	1·09^{b}	0·056	1·01^{b}	0·047	0·77^{a}	0·049	0·67^{a}	0·021	0·89^{a,b}	0·095	< 0·001	0·501	0·032
20 : 2*n*-6†	0·51^{a}	0·021	0·51^{a}	0·020	0·48^{a}	0·019	0·73^{c}	0·019	0·62^{b}	0·019	0·68^{b,c}	0·019	< 0·001	0·007	0·017
20 : 3*n*-6†	0·04^{a}	0·004	0·04^{a,b}	0·004	0·05^{a,c}	0·005	0·08^{d}	0·007	0·06^{c,d}	0·003	0·06^{b,c}	0·004	< 0·001	0·110	0·001
20 : 3*n*-3	0·12^{a}	0·004	0·14^{a}	0·006	0·11^{a}	0·006	0·17^{b}	0·009	0·13^{a}	0·008	0·14^{a,b}	0·011	0·003	0·020	0·002
22 : 4*n*-6†	0·07	0·011	0·08	0·007	0·07	0·005	0·25	0·019	0·21	0·011	0·24	0·024	< 0·001	0·365	0·217
Others	0·29	0·015	0·25	0·009	0·25	0·021	0·42	0·042	0·38	0·024	0·34	0·046	< 0·001	0·214	0·742
Fatty acid partial sums															
SFA†	43·5	0·67	44·0	0·64	44·2	0·61	41·1	0·61	42·5	0·61	41·3	0·61	< 0·001	0·368	0·495
MUFA	45·3	0·68	45·2	0·48	45·2	0·56	41·0	0·63	40·6	0·57	43·0	0·89	< 0·001	0·194	0·188
PUFA†	11·3	0·26	10·6	0·25	10·3	0·22	17·5	0·49	16·5	0·33	15·4	0·69	< 0·001	0·003*	0·480
n-6 PUFA†	10·4	0·24	9·72	0·230	9·47	0·201	16·3	0·46	15·5	0·31	14·4	0·64	< 0·001	0·004*	0·479
n-3 PUFA†	0·92	0·018	0·86	0·021	0·81	0·026	1·22	0·034	1·08	0·025	1·02	0·047	< 0·001	< 0·001*	0·249
Fatty acid ratios															
PUFA:SFA†	0·26	0·010	0·24	0·007	0·23	0·007	0·43	0·016	0·39	0·011	0·37	0·018	< 0·001	0·006*	0·611
n-6:*n*-3	11·3	0·16	11·4	0·11	11·7	0·22	13·4	0·19	14·3	0·19	14·1	0·17	< 0·001	0·005**	0·058

a,b,c,d Mean values within a row with unlike superscript letters were significantly different (P < 0·05).
* Control > RPDL, control > RPD, RPDL = RPD.
† Variable adjusted for breed × diet × P_2 interaction.
‡ Variable adjusted for breed × P_2 interaction.
§ Variable adjusted for P_2.
‖ Variable adjusted for diet × P_2 interaction.
¶ Control = RPDL, control > RPD, RPDL = RPD.
** Control < RPDL, control < RPD, RPDL = RPD.

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P_2 backfat thickness, which is the most representative location[36], a significant effect of breed ($P<0·001$) was observed, in contrast to the diet ($P=0·318$), with values for Alentejano pigs being 90 % higher than those obtained for crossbred animals. Similar values for backfat thickness in relation to dietary treatment were obtained at shoulder, last lumbar vertebra and second sacral vertebra locations (data not shown). Regarding the total fatty acids (expressed as percentage of SAT weight), a significant effect of breed ($P<0·001$), with the highest values for Alentejano pigs, was observed. In contrast to backfat thickness, the percentage of total fatty acids in SAT was higher ($P=0·049$) under the RPD by 2–4 %, when compared with the control diet.

The most abundant fatty acids in SAT were 18 : 1*c*9 (30–35 % of total FAME), 16 : 0 (24–26 %), 18 : 0 (15–16 %), 18 : 2*n*-6 (9–15 %) and 18 : 1*c*11 (7–8 %), in all the groups investigated. The breed and diet interaction influenced six fatty acids, including the major SFA 16 : 0. The breed affected seven of the eighteen fatty acids identified and the 'others' detected fatty acids. The proportions of the major fatty acids 18 : 1*c*9 ($P<0·001$) and 18 : 2*n*-6 ($P<0·001$) were the highest in Alentejano and crossbred pigs, respectively. In SAT, the diet affected only three fatty acids. The proportions of 16 : 1*c*7, 18 : 2*n*-6 and 18 : 3*n*-3 were lower in the RPD than in the control diet.

All partial sums of fatty acids and both fatty acid ratios (PUFA:SFA and *n*-6:*n*-3) were strongly affected ($P<0·001$) by breed (Table 4). As a consequence of the breed effect on individual fatty acids, the partial sums of SFA and MUFA were higher in Alentejano animals, whereas PUFA, *n*-6 PUFA, *n*-3 PUFA and both fatty acid ratios were higher in crossbred pigs. The RPD decreased PUFA ($P=0·003$), *n*-6 PUFA ($P=0·004$), *n*-3 PUFA ($P<0·001$) and PUFA:SFA ratio ($P=0·006$) when compared with the control diet. In contrast, the *n*-6:*n*-3 ratio was increased under the RPD ($P=0·005$) when compared with the control diet.

Gene expression levels of muscle and subcutaneous adipose tissue

The results previously described demonstrate different responses of the longissimus lumborum muscle and SAT under RPD in the crossbred pigs, but not in the Alentejana breed. In order to investigate the mechanism underlying the genotype- and tissue-specific effects of the diets, an assessment of expression of key genes associated with lipid metabolism was carried out. The expression levels of ten key genes controlling lipid metabolism has been analysed in longissimus lumborum muscle and SAT, and the results are presented in Figs. 1 and 2, respectively.

The expression patterns of the genes in longissimus lumborum muscle were similar ($P>0·05$) across all dietary treatments, with an exception for *PPARG* ($P=0·016$) (Fig. 1). The *PPARG* mRNA levels were higher in the crossbred pigs when compared with the Alentejana breed. The relative expression levels of the genes investigated in the longissimus lumborum muscle was not affected by the dietary protein content ($P>0·05$). However, an interaction between breed and diet ($P=0·018$) was observed for *SCD* mRNA in muscle, with the *SCD* expression increased under the RPD in crossbred animals but not in Alentejano pigs.

In SAT, relative *CEBPA* ($P<0·001$), *CRAT* ($P=0·01$), *PPARA* ($P=0·008$) and *SCD* ($P<0·001$) mRNA levels were higher in the Alentejano animals when compared with the crossbred pigs (Fig. 2). In contrast to genotype, diet did not affect the expression level of any of the genes investigated in SAT. There was a breed and diet interaction for the mRNA levels of *ACACA* ($P=0·044$), *FABP4* ($P<0·001$), *FASN* ($P=0·049$) and *LPL* ($P=0·009$) genes in SAT. The expression level of *ACACA* and *LPL* genes were down- and up-regulated, respectively, by the RPD in the Alentejano pigs. However, these variables were not affected in the crossbred animals. The mRNA levels of *FABP4* were increased under the RPD diet in crossbred pigs and decreased in Alentejano pigs. Finally, the ratio between muscle and SAT *LPL* gene expression (muscle: SAT ratio) was higher ($P=0·045$) in crossbred pigs than in Alentejano animals.

Correlation between fatty acid composition and gene expression levels

The correlation coefficients (r) between fatty acid composition and gene expression levels, adjusted for IMF as covariate for longissimus lumborum muscle and for P_2 backfat thickness as covariate for SAT, are shown in Table 5. In longissimus lumborum muscle, the 16 : 1*c*9 ($P<0·001$), MUFA ($P<0·01$), *PPARG* ($P<0·01$), *PPARA* ($P<0·05$) and *FABP4* ($P<0·01$) were positively and moderately correlated ($0·7 \geq r \geq 0·3$) with *CEBPA*. 18 : 2*n*-6 and PUFA were positively correlated with *CRAT*, while MUFA was negatively correlated with the same gene. The *LPL* and *SCD* genes were not correlated with any fatty acid.

In SAT, 18 : 1*c*9 and MUFA were positively and moderately correlated with most of the genes, in contrast to 18 : 2*n*-6 and PUFA, which were negatively correlated. *SCD* was positively correlated with the 18 : 1*c*9 ($P<0·001$) and MUFA ($P<0·001$) and negatively correlated with 18 : 2*n*-6 ($P<0·001$) and PUFA ($P<0·001$).

Discussion

The increased IMF obtained in the present study for growing crossbred (lean) pigs fed a 25 % RPD (17·5 *v.* 13·1 % of crude protein) and not equilibrated for lysine, is in agreement with several previous studies using a range of protein concentrations (e.g. 20 *v.* 16 %[12]; 21 *v.* 18 %[9]). However, whether the muscle lipogenic response was due to the reduction of dietary protein *per se*, decrease of dietary lysine level or both remains to be established. Alonso *et al.*[37] observed an increase in IMF content (from 1·8 to 2·6 % in the muscle) under the RPD (from 17 to 15 %) but with similar dietary lysine contents (0·8 %). In contrast, in our previous study in the traditional Bizaro pig breed (RJB Bessa, unpublished results), where we tested the effect of the reduction of dietary protein from 17 to 14 % at constant lysine levels, no significant increase of IMF was obtained. This is not in line with reports

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of other authors, who observed a negative relationship between dietary lysine and IMF content[38]. Furthermore, D'Souza *et al.*[10] reported that pigs fed a diet with a 15 % reduced lysine:energy ratio in the diet during the growing phase had higher IMF levels. This discrepancy of data might be due to the use of different pig genotypes. In fact, based on studies with two modern (Duroc and Large White breeds) and two traditional (Berkshire and Tamworth breeds) pig genotypes, Wood *et al.*[39] suggested that the mechanisms regulating fat partitioning are genotype specific. Therefore, it was important to undertake a comprehensive study on the effect of both low dietary protein and lysine levels on fat partitioning in diverse pig genotypes.

The present study addressed the aforementioned aspects and demonstrated that the only dietary treatment that increased IMF in longissimus lumborum muscle was the RPD and not the RPDL (RPD equilibrated for lysine) in crossbred pigs. Thus, the present results clearly indicate that it is the reduction of lysine availability in the diet that promotes IMF deposition in lean pig genotypes. An additional important finding of the present study is that the responses to the dietary treatments (reduction of protein or lysine) depended on the pig breed. Thus, the IMF of Alentejana purebred (fatty) pigs in the control group was 155 % higher than that of the commercial crossbred (lean pigs). In contrast

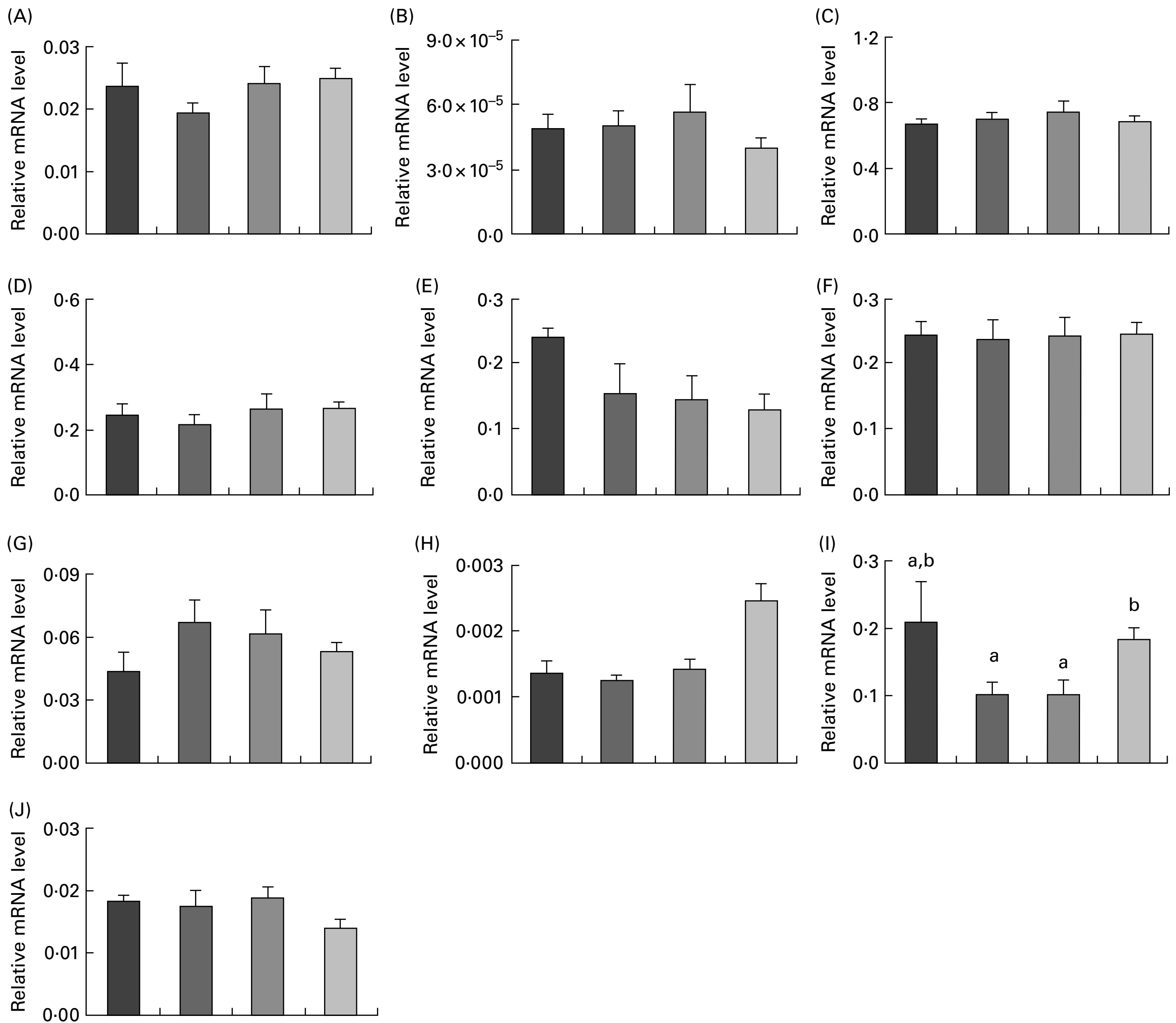

Fig. 1. Effect of the reduced protein diet (RPD) not equilibrated for lysine level on gene expression levels in longissimus lumborum muscle of Alentejana purebred and crossbred pigs: (A) acetyl-CoA carboxylase (ACACA), (B) CCAAT/enhancer binding protein-α (CEBPA), (C) carnitine *O*-acetyltransferase (CRAT), (D) fatty acid-binding protein 4 (FABP4), (E) fatty acid synthase (FASN), (F) lipoprotein lipase (LPL), (G) PPAR alpha (PPARA), (H) PPAR gamma (PPARG) (breed, $P = 0.016$), (I) stearoyl-CoA desaturase (SCD) (breed × diet, $P = 0.018$), (J) sterol regulatory element-binding protein 1 (SREBP1). Values are means, with their standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P < 0.05$). 'Breed' and 'breed × diet' mean significant effect of breed or interaction between breed and diet, respectively. For ACACA, CEBPA, variable adjusted for diet × IMF interaction. For FABP4, PPARG, variable adjusted for breed × IMF interaction. ■, Alentejana–control diet; ■, Alentejana–RPD; ■, crossbred–control diet; ■, crossbred–RPD.

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to the crossbred animals, Alentejano animals did not respond to any dietary treatment.

It is well-known that lysine is often the limiting amino acid for the growing rate of pigs fed cereal-based diets[40] and that low dietary protein and lysine levels limit protein synthesis and increase the energy available for fat deposition, with the consequent increase in IMF[36]. The Alentejana breed, which is similar to the Spanish Iberian breed, has a low capacity for lean tissue deposition[41] and thus lower dietary lysine requirements. Therefore, the absence of effects of RPD in Alentejana breed pigs is possibly due to the fact that lysine did not limit protein deposition.

Another possible explanation for the distinct response of the two pig genotypes to the RPD in the present experiment might be the genotype-specific expression of key lipogenic enzymes. In fact, it has been previously demonstrated that the expression of lipogenic enzymes, mainly SCD, have a critical impact on IMF deposition in pigs[42]. In the present study, *SCD* gene expression was increased under the RPD in crossbred but not in Alentejano pigs. In addition, the expression of the key transcription factor controlling lipid metabolism, PPARG, showed a similar trend. This is consistent with the findings of Guo *et al.*[42], who observed an increase of the muscle *PPARG* mRNA levels in the muscle, but not in

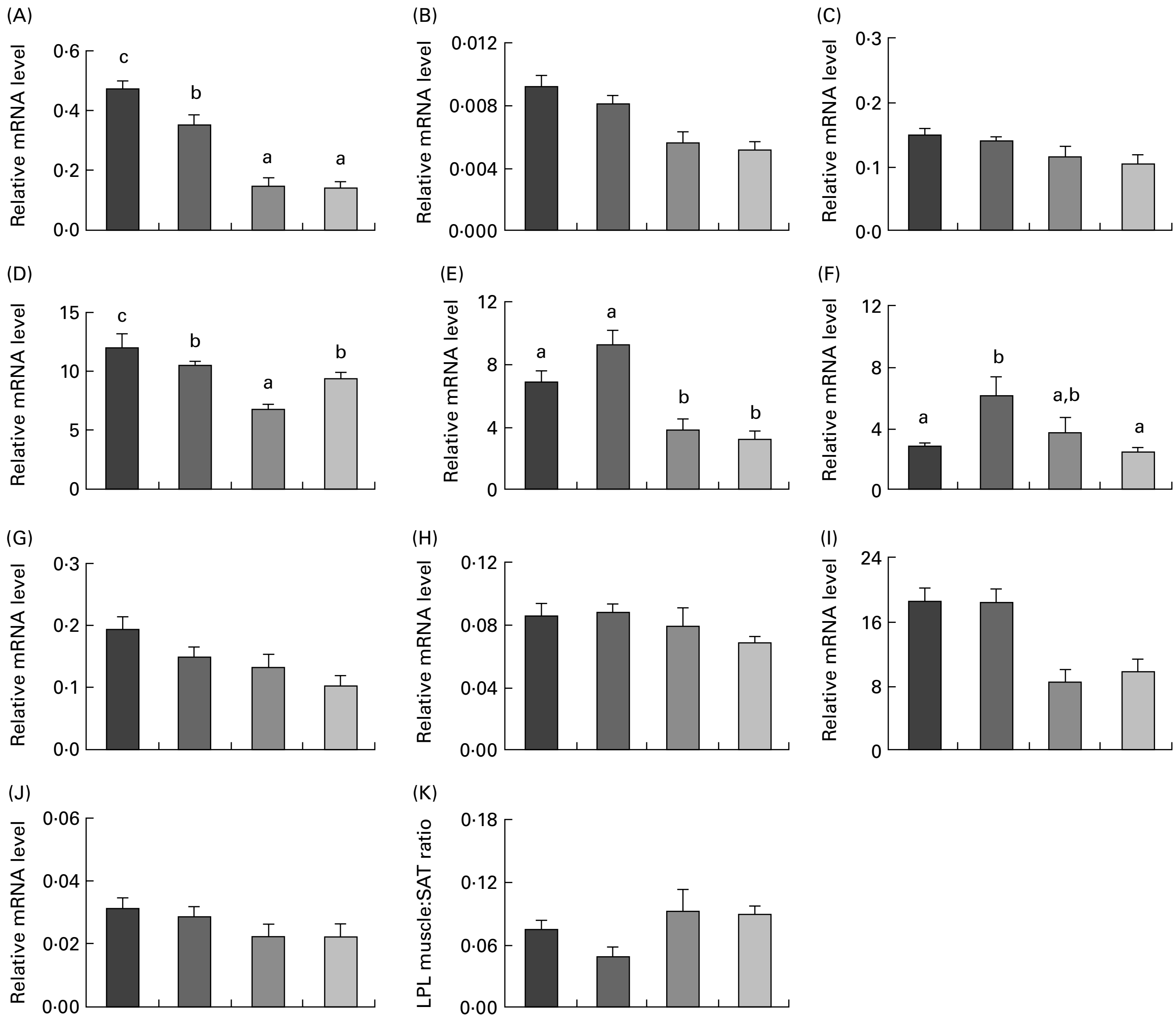

Fig. 2. Effect of the reduced protein diet (RPD) not equilibrated for lysine level on gene expression levels in subcutaneous adipose tissue of Alentejana purebred and crossbred pigs: (A) acetyl-CoA carboxylase (ACACA) (breed × diet, P=0·044), (B) CCAAT/enhancer binding protein alpha (CEBPA) (breed, P<0·001), (C) carnitine *O*-acetyltransferase (CRAT) (breed, P=0·010), (D) fatty acid-binding protein 4 (FABP4) (breed × diet, P<0·001), (E) fatty acid synthase (FASN) (breed × diet, P=0·049), (F) lipoprotein lipase (LPL) (breed × diet, P=0·009), (G) PPAR alpha (PPARA) (breed, P=0·008), (H) PPAR gamma (PPARG), (I) stearoyl-CoA desaturase (SCD) (breed, P<0·001), (J) sterol regulatory element-binding protein 1 (SREBP1), (K) LPL muscle/subcutaneous adipose tissue (SAT) (breed, P=0·045). Values are means, with their standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different (P<0·05). 'Breed' and 'breed × diet' mean significant effect of breed or interaction between breed and diet, respectively. For FABP4, variable adjusted for breed × diet × P_2 (last rib position) interaction. ■, Alentejana–control diet; ■, Alentejana–RPD; ■, crossbred–control diet; □, crossbred–RPD.

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Table 5. Pearson's correlation coefficients among total fatty acids (g/100 g subcutaneous adipose tissue), fatty acid composition (% total fatty acids) and gene expression levels (relative mRNA level) in the longissimus lumborum muscle and subcutaneous adipose tissue of Alentejana purebred and crossbred pigs

	16:0	16:1*c*9	18:0	18:1*c*9	18:1*c*11	18:2*n*-6	SFA	MUFA	PUFA	*SREBP1*	*SCD*	*PPARG*	*PPARA*	*LPL*	*FASN*	*FABP4*	*CRAT*	*CEBPA*	*ACACA*
Longissmus lumborum muscle																			
ACACA	−0·17	0·23	−0·24	−0·24	0·44**	0·07	−0·24	−0·04	0·10	0·18	0·63***	0·63***	0·38*	0·33*	−0·07	0·61***	0·15	0·29	
CEBPA	0·01	0·50***	−0·26	0·25	0·30	−0·37*	−0·18	0·43**	−0·33*	0·13	0·12	0·45**	0·34*	0·28	−0·21	0·47**	0·13		
CRAT	−0·16	−0·02	−0·12	−0·38*	−0·06	0·40**	−0·18	−0·48**	0·43**	0·12	−0·08	−0·03	−0·21	0·36*	0·28	−0·04			
FABP4	−0·16	0·30	−0·23	−0·03	0·38*	−0·07	−0·19	0·25	−0·03	0·23	0·44**	0·57***	0·30	0·56***	−0·03				
FASN	0·23	−0·20	0·39*	−0·08	−0·17	−0·00	0·37*	−0·17	−0·03	0·02	0·19	−0·14	−0·72***	−0·04					
LPL	−0·09	−0·06	−0·10	−0·08	−0·07	0·17	−0·05	−0·11	0·17	0·22	0·27	0·14	0·25						
PPARA	0·02	0·31*	−0·23	−0·00	0·24	−0·07	−0·10	0·20	−0·07	0·17	0·17	0·33*							
PPARG	−0·23	0·48*	−0·40*	−0·04	0·60***	−0·07	−0·34*	0·30	−0·04	−0·18	0·34*								
SCD	0·20	−0·08	0·17	0·16	−0·12	−0·18	0·23	0·10	−0·17	0·10									
SREBP1	−0·06	−0·04	−0·01	−0·05	0·02	0·03	−0·06	−0·08	0·03										
Subcutaneous adipose tissue																			
TFA	0·17	0·04	0·02	0·02	−0·11	−0·07	0·12	0·00	−0·06	0·09	0·00	−0·23	−0·08	0·02	−0·03	−0·09	−0·26	−0·16	−0·09
ACACA	0·08	−0·11	0·23	0·61***	−0·22	−0·65***	−0·07	0·41**	−0·65***	0·55***	0·84***	0·53***	0·69***	0·22	0·69***	0·63***	0·60***	0·75***	
CEBPA	0·05	−0·20	0·08	0·64***	−0·19	−0·58***	−0·07	0·53***	−0·58***	0·62***	0·79***	0·39*	0·76***	0·25	0·58***	0·52***	0·61***		
CRAT	0·06	0·23	−0·21	0·48**	−0·08	−0·38*	−0·16	0·50**	−0·38*	0·53***	0·64***	0·50**	0·61***	0·32*	0·61***	0·40*			
FABP4	−0·37*	−0·26	0·11	0·64***	0·14	−0·61***	−0·50***	0·57***	−0·61***	0·20	0·51***	0·18	0·34*	−0·09	0·40*				
FASN	0·33*	−0·07	0·27	0·63***	−0·47**	−0·62***	0·16	0·33*	−0·62***	0·37*	0·71***	0·33*	0·56**	0·39*					
LPL	0·29	0·20	−0·07	0·33*	−0·49**	−0·21	0·19	0·21	−0·21	0·05	0·18	0·09	0·20						
PPARA	0·02	−0·06	0·01	0·40**	−0·08	−0·36*	−0·07	0·34*	−0·36*	0·73***	0·76***	0·52***							
PPARG	0·16	0·17	0·07	0·17	−0·14	−0·21	0·05	0·07	−0·22	0·20	0·41**								
SCD	0·05	−0·03	0·10	0·65***	−0·17	−0·70***	−0·06	0·56***	−0·70***	0·76***									
SREBP1	0·05	−0·06	−0·02	0·27	0·13	−0·37*	−0·03	0·40*	−0·36*										

SREBP1, sterol regulatory element-binding protein 1; SCD, stearoyl-CoA desaturase; PPARG, PPAR-γ; PPARA, PPAR-α LPL, lipoprotein lipase; FASN, fatty acid synthase; FABP4, fatty acid-binding protein 4; CRAT, carnitine *O*-acetyltransferase; CEBPA, CCAAT/enhancer binding protein alpha; ACACA, acetyl-CoA carboxylase.

* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.

SAT, of crossbred pigs fed high-energy low-protein diets. The present study did not find any significant effect of diet on the mRNA level of other key genes controlling fatty acid deposition, such as *ACACA*, *CEBPA*, *CRAT*, *FABP4*, *FASN*, *LPL*, *PPARA* and *SREBP1*. The present results are consistent with the findings of Gondret & Lebret[43], who described that ACACA activity in the longissimus muscle of pigs does not respond to feeding manipulation, including protein- and energy-restricted diets. In contrast, Damon *et al.*[44] reported an association between IMF and FABP4 in pigs, but his study was focused on protein expression, whilst the present study investigated the mRNA content. In fact, it is well-known that changes in protein expression are not always preceded by changes in mRNA expression[45].

The present study showed that the RPD increased 16:0 and SFA proportions and reduced the proportions of PUFA in the longissimus lumborum muscle of both pig breeds. These results are in agreement with those reported by Teye *et al.*[36], who observed that low-protein and -lysine diets (21 and 1 % *v.* 18 and 0·7 % of protein and lysine, respectively) in Duroc × Large White × Landrace crossbred pigs decreased total PUFA proportions. This effect could be a result of the distinct distribution of fatty acids between TAG (richer in SFA and MUFA) and phospholipids (richer in PUFA) and the increasing proportion of TAG with increasing IMF content[46]. Although the increased *SCD* mRNA expression in crossbred pigs fed the RPD suggests an enhanced SCD activity, the 18:1*c*9 proportion did not confirm this hypothesis. In addition, it was previously proposed by Doran *et al.*[9] that RPD increased the IMF in pigs due to both the activation of protein expression and increased activity of SCD. Furthermore, Ntawubizi *et al.*[46] found that IMF content was positively related to SCD and elongase activities in the longissimus muscle. It is well-known that in monogastric animals, fatty acid composition can be strongly influenced by dietary factors. However, the dietary factors can be diluted by *de novo* SFA and MUFA biosynthesis, thus resulting in a decline of the PUFA:SFA ratio with increasing fat deposition[47]. The *n*-3 PUFA proportions in the present study were very low, which could be explained by low levels of *n*-3 fatty acids in cereal-based diets and is very undesirable from a human nutrition perspective[48].

As expected, in the present study, backfat thickness was higher in the Alentejano (fatty) pigs than in the crossbred (lean) animals. Moreover, the content of total fatty acids in SAT, expressed on a tissue weight basis, was also higher in the Alentejano pigs. FABP4 protein is known to be responsible for the transport of fatty acids in adipocytes and its content is associated with backfat thickness[49] and IMF content[44]. Furthermore, Hocquette *et al.*[11] suggested that FABP4 protein can be used as a marker of adipocyte number in tissues. The present study showed that *FABP4* mRNA level in SAT was 40 % greater in the control group of the Alentejana breed when compared with the crossbred pigs. This is in agreement with the greater backfat thickness of the carcasses of Alentejano pigs. In addition, the up-regulation of *FABP4* gene in crossbred pigs fed with the RPD, when compared with the control crossbred pigs, is consistent with the higher content

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of total fatty acids in the SAT. Interestingly, the findings were different in the muscle. In spite of the higher level of IMF in Alentejano pigs, and increased IMF under the RPD in the crossbred pigs, the *FABP4* mRNA expression was not affected either by breed or the diet. The genotype differences in the SAT fatty acid content and composition reported in the present study may be explained by a higher expression of the genes controlling lipogenesis (*ACACA*, *FASN* and *SCD*) and expression of the transcription factor *CEBPA*. This suggestion is in line with results of De Pedro[50], who reported that carcasses of Iberian pigs, a genotype similar to Alentejana purebred, have higher fatty acid contents than commercial crossbred genotypes in the SAT. The higher *FASN* expression level in SAT of Alentejano pigs, when compared with the crossbred animals, observed in the present study, is consistent with the higher 16 : 0 proportion in SAT of Alentejano pigs, as 16 : 0 is the end product of *de novo* synthesis of SFA.

In addition to the genotype-specific response to the RPD, another important finding of the present study is the tissue-specific effect of the same diet. The crossbred pigs demonstrated a large increase in fat content in muscle (55 %), with only a small increase in total fatty acids content in SAT (4 %) under the RPD. Furthermore, tissue-specific responses to RPD were also observed in the mRNA expression patterns for *ACACA*, *FASN* and *SCD*. This is in line with the results of Doran *et al.*[9], who observed significant differences in responses of muscle and SAT fatty acid composition and SCD to an RPD. This was tentatively explained by tissue-specific expression of SCD isoforms. In adipose tissue, which is the main site for *de novo* fatty acid synthesis in pigs[51,52], SCD activity was apparently not affected by the RPD because the percentages of 16 : 1*c*9 and 18 : 1*c*9 and SCD mRNA levels did not change. Thus, it is very unlikely that SCD activity could increase with low-protein diets in this tissue. In addition, intramuscular adipose tissue, the last fat depot to develop, may respond to dietary conditions in a different manner from other fat sites[43].

Previous gene expression profiling and proteomics studies suggested that pathways involved in lipid and energy metabolism are clearly down-regulated in intramuscular adipocytes when compared with fat cells from other depots[53–55]. In the present study, regardless of the breed or diet, mRNA levels of *ACACA*, *FASN*, *FABP4*, *PPARG*, *LPL*, *CEBPA*, *SCD* and *SREBP1* were higher in SAT than in muscle. Also, major fatty acids and partial sums of fatty acids were much more correlated with the expression level of key lipogenic enzymes and transcription factors in SAT than in muscle. Although muscles contain a relatively low proportion of adipocytes, some authors found that mRNA levels of genes related to lipid metabolism were lower in intramuscular adipocytes than in subcutaneous adipocytes[56]. However, in the present study, the expression level of *CRAT* was lower in SAT than in muscle, thus suggesting a higher activity of β-oxidation of fatty acids in muscle than in SAT. Although CEBPA, PPARG and SREBP1 are key regulators of adipogenesis, it was suggested that SREBP1 is a transcription factor induced during the early stages of adipogenesis, inducing the expression of CEBPAα and PPARAγ only in the later phases of fat deposition[57]. This may explain the absence of genotype differences or responses of this adipogenic factor to dietary treatment in the present study. This suggestion is in line with the findings by Ding *et al.*[58], who demonstrated that SREBP1 mRNA expression in adipose tissue does not differ between Newsham-sired and Duroc-sired pigs, suggesting that genetic selection does not affect the expression of the aforementioned gene.

LPL is a rate-limiting enzyme responsible for hydrolysis of circulating TAG carried out in VLDL and chylomicrons and is, generally, produced primarily by muscle and mature adipocytes[59]. Therefore, LPL modulates partitioning of fatty acids between oxidation in skeletal muscle and storage in white adipose tissue[60]. The results of the present study showed that *LPL* mRNA was expressed at higher levels in SAT, when compared with the muscle (*LPL* muscle:SAT ratios $<0{\cdot}1$). This suggests that circulating fatty acids were mainly used for storage in SAT. In addition, the higher *LPL* muscle:SAT ratios in the crossbred pigs, when compared with the Alentejano animals, indicate a lower storage capacity of SAT in the crossbred pigs. Interestingly, plasma concentrations of TAG (318 *v.* 388 mg/l, $P=0{\cdot}002$, for the control diet and RPD, respectively) and NEFA (46 *v.* 68 μmol/l, $P=0{\cdot}038$, for the control diet and RPD, respectively) in Alentejano pigs had a similar pattern to that of *LPL* gene expression in SAT, i.e. with increased values for the RPD when compared with the control diet. Therefore, it is possible that once NEFA are released from VLDL by the action of LPL, they are taken up mainly by the adipocytes of SAT, thus increasing the mass of this fat depot in Alentejana breed pigs.

Conclusions

To the best of our knowledge, the present study is the first report that demonstrated that RPD increase the IMF content in lean but not in fatty pig breeds. Furthermore, the present results strongly suggest that the increased IMF deposition in lean pig breeds is due to a limitation of lysine level in the diets. Analyses of mRNA expression suggest that the genotype-specific effect of the RPD on IMF content is mediated via up-regulation of the expression of lipogenic enzyme SCD and the adipogenic transcription factor *PPARG*. The muscle fatty acid composition was more affected in lean than in fatty pigs under the RPD, which may be reflected in the change in the TAG:phospholipid ratio, as result of the increased IMF in the former genotype.

Furthermore, the present results indicate that feeding a RPD does not change the backfat thickness, but results in an increase in total fatty acid content in both lean and fatty pig genotypes. When backfat thickness was compared between the control groups of both genotypes, it was established that the higher backfat thickness of fatty pigs, when compared with the lean ones, is associated with higher mRNA levels of the key lipogenic enzymes and transcription factors (*ACACA*, *CEBPA*, *FASN* and *SCD*). Therefore, we can conclude that the fatty acid composition of SAT seems to be more affected by the breed than by the diet, under these experimental conditions.

Overall, the results strongly suggest that adipogenesis and lipogenesis are regulated differently in the longissimus

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lumborum muscle and SAT of pigs, and that this modulation is genotype specific. These findings could help in the development of effective genotype-specific feeding strategies in order to improve fat partitioning in pigs. This insight into the molecular mechanisms underlying regulation of the amount and composition of IMF in pigs may contribute to the development of strategies to satisfy consumers' expectations and to enhance the competitiveness of the meat industry.

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