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Gene expression analysis by RNA-sequencing of *Longissimus dorsi* muscle of pigs fed diets with differing lipid contents

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ABSTRACT. We examined the effect of lipid content in the diet on the transcriptome of the *Longissimus dorsi* muscle in pigs. Our objective was to examine changes at the molecular level affecting economically relevant meat quality characteristics such as intramuscular fat deposition and fatty acid profile. The treatments consisted of isoproteic and isoenergetic diets with differing lipid contents due to addition of rice bran. The control diet (T0) was a normal basic diet and the test diet (T15) had 15% rice bran. The final lipid content (ether extract) in the diets was 3.4 and 4.8% in T0 and

T15, respectively. Three male piglets of the Uruguayan creole breed Pampa Rocha were used per treatment, which lasted from weaning at 42 days until 77 days of age. The animals were reared in confinement on deep bedding and were slaughtered at the end of the experiment, when muscle samples were collected. Intramuscular fat content (IMF) and fatty acid composition were analyzed to determine if diets had a phenotypic effect. Gene expression analysis was performed with RNA-seq methodology to carrying out a functional analysis of genes with differential expression between treatments. The added fat to the diet did not affect IMF or fatty acid composition. However, we identified 359 genes with differential expression between treatments. These genes participate in various metabolic pathways, some of them affecting meat quality. The most relevant genes identified in this regard were *PDK4* (up-regulated with T15), which is associated with energy metabolism, *FOS, ATF3, MYOD1* and *MAFF* (all down-regulated with T15), which are associated with skeletal muscle growth, and *TNC* (up-regulated with T15), which is associated with extracellular matrix-receptor interactions. This study of the skeletal muscle transcriptome in pigs can help understand the genetic basis of how diet affects important production traits.

Key words: Nutrigenomics; Transcriptomic; Skeletal muscle; Candidate genes; Meat quality

INTRODUCTION

Different production factors can influence meat quality, with food being one of the most important factors in pork production. In recent years, there has been a growing interest in the modification of the fatty acid composition of adipose tissue from production animals through diet (Wood et al., 2008). Pigs store dietary fat almost unchanged; thus, there is a close relationship between the type of fat ingested and the fat deposited (Rosenvold and Andersen, 2003). This could affect meat attributes such as intramuscular fat content and composition.

Nutritional requirements for pigs depend on various factors, such as sex, age, physiological state and environmental conditions (Beyli et al, 2012; García-Contreras et al., 2012). As for the lipid content (ether extract), in the case of animals with a body weight between 20-60 kg, it an inclusion range from 4 to 8% is recommended (FEDNA, 2013); although some researchers have indicated values outside this range (Mendoza et al., 2014; Pavéz González, 2014).

Feed accounts for approximately 70% of total pig production costs. Therefore, changes in feed costs have a direct impact on profitability, leading to a search for lower food costs but without affecting production parameters of animals (Bauza, 2000a). This is particularly important for low income rural producers. Rice bran could be considered as an alternative for the formulation animal feed (Barlocco, 2013). In Uruguay, this is a low-cost, easy-to-obtain industrial by-product, as the rice industry is one of the country's main economic activities (DIEA, 2018). From a nutritional point of view, rice bran has a high

energy concentration, and adequate amounts of protein, on the order of 10 to 15%. It is characterized by a high content of unsaturated fatty acids, which may affect the oxidative stability of meat (Cozzolino, 2000). However, its inclusion in diets may be beneficial if the meat is intended for fresh consumption (Bauza et al., 2007), particularly considering nutritional recommendations for humans that promote an increase in polyunsaturated fatty acid consumption (Simopoulos, 2016). This ingredient has already been incorporated into commercial hybrid pig diets in experimental trials, including the work of Capra et al. (2011) and Bauza et al. (2007).

Nutrigenomics provides a means to investigate the influence of dietary nutrients. This discipline studies the role of nutrients and bioactive components of food on gene expression, including their effect on genome stability, RNA expression, proteins, and metabolites (Fenech et al., 2011). Kato and Kimura (2003) argue that the study of transcriptomes, the complete set of RNA molecules expressed in an organism, cell type or tissue under certain conditions, constitutes one of the levels where modifications in response to nutrients can be used as a global marker of the status and response of cells and organs to modifications at other molecular levels, such as the proteome and metabolome. The introduction of new generation RNA sequencing technology (RNA-seq) has revolutionized transcriptomic studies as a result of increased knowledge on quantitative and qualitative aspects of transcriptomes (Loor et al., 2015), leading in recent years to an increase in the studies that use this technology for the analysis of the transcriptome in different species. In the case of pigs, various studies have evaluated the effect of feed ingredients on the transcriptome, particularly of skeletal muscle, by RNA-seq (Szostak et al., 2016; Ogłuszka et al., 2017). The study of variations in gene expression under certain conditions in pigs is useful for interpreting functional elements of the genome and improving the understanding of complex traits, such as fat deposition, metabolism, and growth (Chen et al., 2011). Another factor affecting meat quality, including intramuscular fat content and composition, is the genetic type of pig. While commercial breeds are better suited for intensive production systems and have better growth rates compared to local breeds; the latter have not been subjected to severe selection pressures and in many cases are known for production of high quality meat (Ayuso et al., 2016). The local Uruguayan Pampa Rocha breed has high genetic variability (Montenegro et al., 2015), standing out for its prolificacy and the oxidative stability and acceptable quality for industrialization of its meat (Vadell et al., 2010; Carballo, 2013).

The study of the effects of diet on gene expression of skeletal muscle allows us to understand the changes at the molecular level affecting important production traits. Likewise, if we consider that the pig is one of the most important species in biomedicine, this type of research can provide relevant information about the genes associated with energy metabolism diseases in humans. We exameind effect of diets with different lipid content on skeletal muscle transcriptome through RNAseq in piglets of the local Pampa Rocha breed. The ingredient chosen to increase the lipid content was rice bran, due to its nutritional characteristics, low cost, and availability in Uruguay. Through this approach, we intended to characterize gene expression as a result of these diets and determine how they affect biological processes related to intramuscular fat deposition and fatty acid profile in the meat.

MATERIAL AND METHODS

Animals, treatments, and phenotypic determinations

The procedures with animals were performed according to protocol No. Exp. 111130-000834-13, approved by the Honorary Commission of Animal Experimentation (CHEA, Udelar, Uruguay). Six male piglets of the Pampa Rocha breed, three per treatment, were weaned at 42 days with an average initial weight of 14.85 ± 1.93 kg. The experiment lasted 35 days and was carried out in a 50 cm deep bed system of wheat straw. The animals were housed in confinement with a 0.52 m^2/ animal surface, with group feeders, and drinking troughs to allow free access to water.

We used as treatments isoproteic and isoenergetic conventional diets manually prepared, with different lipid content determined by the inclusion of rice bran in one of the treatments. For the T0 treatment, we used a diet made from the two most conventional foods worldwide used in pig feed: maize grain and soybean meal. The formulation of diets containing these main foods is possible if they are supplemented with a vitamin-mineral premix (NRC, 1998). The percentage composition of ingredients of the control diet (T0) was: 68% ground maize, 28.5% soybean meal, 1.72% dicalcium phosphate, 0.8% calcium carbonate, 0.5% vitamin mineral premix, 0.5% sodium chloride and 0% rice bran.

The composition of the diet with higher lipid content (T15) was: 55% ground maize, 26.5% soybean meal, 1.72% dicalcium phosphate, 0.8% calcium carbonate, 0.5% vitamin mineral premix, 0.5% sodium chloride and 15% rice bran. The final lipid content (ether extract) in the diets was 3.4 and 4.8% in T0 and T15, respectively. In T15, the ether extract is in the range recommended by FEDNA (2013), while this value was lower in T0 to generate a difference in lipid content.

The composition of premix mineral-vitamin used (per kilogram of product) was: DL methionine 3.3%, Lysine HCL 10.2%, Threonine 2.97%, Calcium 13.4%, Phosphorus 4.5%, Sodium 6.7%, Chlorine 10%, Manganese 1,089 mg, Zinc 2,904 mg, Iron 1,980 mg, Copper 4,851 mg, Iodine 19.8 mg, Selenium 13.2 mg, Vitamin A 222,750 IU, Vitamin D3 52,800 IU, Vitamin E 825 IU, Vitamin K3 99 mg, Vitamin B1 41.25 mg, Vitamin B2 178.2 mg, Vitamin B6 49.5 mg, Niacin 907.5 mg, Ca pantothenic 462 mg, Vitamin B12 1.09 mg, Biotin 1.42 mg, Folic acid 28.05 mg, Natuphos 10.000 2,400 mg, Choline chloride 8,250 mg (Nutritec - Grappiolo y Cia. S.A.).

The rice bran percentage used in treatment T15 corresponds to the maximum tolerated by piglets, according to Bauza (2000b). The chemical composition of the rice bran was: 88.9% dry material, 13.1% crude protein, 10.8% ash, 8.3% crude fiber, and 14.8% ether extract (data on fresh basis and provided by Molino San José S.A., the company that supplies the rice bran).

The energy content in both diets was approximately 3,300 kcal of digestible energy per kg and 18% crude protein (National Research Council Requirements Tables, 1998). Feeding was carried out once a day (8:00 AM), basing the daily food supply on

live weight of animals and considering a 75% restriction concerning the theoretical maximum intake during the first four days.

The animals were slaughtered at the end of the experiment, at 77 days of age and average weight of 33.63 ± 4.11 kg (weight was not affected by the diets: P-value = 0.77 for Student's *t*-Test at a 0.05 confidence level). The *Longissimus dorsi* (LD) muscle was subsequently removed, and samples of 5 g were collected for RNA extraction. These samples were stored in RNA stabilization solution (RNAlater®) at -20°C until the time of extraction.

The differential effect of diets was assessed through calculation of fatty acid content and profile in intramuscular fat. This analysis was performed using the techniques and procedures described in Montenegro et al., (2018), where 12 animals were used, including the six animals analyzed in this paper. Animals were selected according to the quality of the extracted RNA measured through RIN (RNA integrity number) values. Treatment mean comparison of these variables was performed with the Student *t*-Test at a 0.05 confidence level. The analysis was performed with the statistical software Infostat (Di Rienzo et al., 2014).

RNA isolation, library construction, and RNA sequencing

Sample homogenization was performed in a FastPrep®-24 equipment (MP Biomedicals) using MP Bio Lysing Matrix and Trizol® tubes. Total RNA was obtained using Trizol® (Thermo Fisher Scientific®) according to the manufacturer's instructions. Concentration of RNA samples was measured on a Nanodrop ND1000 spectrophotometer. The RNA quality control, library preparation, and sequencing were performed by Macrogen. Briefly, control of total RNA integrity was performed with an Agilent Technologies 2100 Bioanalyzer. Libraries were constructed from total RNA with the TruSeq RNA Sample Prep Kit v2. Sequencing libraries were prepared by random fragmentation of the cDNA sample followed by ligation of 5' and 3' adapters. The adapter-ligated fragments were subsequently amplified by PCR and gel purified. To verify the size of the PCR-enriched fragments, the size distribution of the template was verified with an Agilent Technologies 2100 bioanalyzer. Library quantification was performed through qPCR according to the Illumina qPCR Quantification Protocol Guide. RNAseq was performed on an Illumina HiSeq 2000 platform to generate 100-bp paired-end (Macrogen).

Bioinformatics analysis and functional analysis of differential expression

For the quality control of the sequences and data analysis, the CLC Genomics Workbench 9.5.2 (CLC bio, Aarhus, Denmark) software was used. The reads were mapped onto the porcine reference genome Sus scrofa v.10.2 (GCA_000003025.4) using the default parameters of the RNA-seq analysis of CLC software.

Differential expression analysis was performed using a generalized linear model (GLM), and genes with fold change values ≥ 2 and P-value ≤ 0.05 were considered significant. Differential expression genes (DEGs) were classified into biological

processes (BP), molecular functions (MF), and cellular components (CC) using the Gene Ontology (GO) classification. The identification of significant pathways was performed according to the Kyoto Encyclopedia of Genes and Genomes (KEGG). This analysis was performed, for all DEGs without discriminating their expression level between treatments, on the "Database for Annotation, Visualization and Integrated Discovery" (DAVID) v.6.8 (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009a; 2009b). The DEGs were introduced using the official gene symbol and human genome annotation through the BioMart tool. Terms and pathways with a P-value ≤ 0.05 for Fisher's Exact Test (EASE score) with Benjamini correction were considered significant.

Additionally, we analyzed the relationships between up-regulated and down-regulated genes in T15 compared to T0, using the ClueGo v 2.3.3 application, available at Cytoscape v. 3.4.0 (Bindea et al., 2009; Saito et al., 2012). In this way, *Homo sapiens* annotation and the BP of GO and KEGG databases were used. Probability was calculated through the hypergeometric test (two-sided) with the Benjamini correction. Three genes per node were used as parameters, with a minimum percentage of associated genes of 3% and a Kappa score of 0.4.

RESULTS

Intramuscular fat content and composition of fatty acids

There were no significant differences in these variables. We observed a higher content of polyunsaturated fatty acids, more specifically of linoleic acid, in the samples of animals treated with the diet with higher lipid content. The values found were 16.52 and 13.61% of polyunsaturated fatty acids (PUFA); and 13.8 and 11.27% of linoleic acid in T15 and T0 respectively. These results are available in the Supplementary material (Supplementary 1).

Analysis of data obtained by RNA-seq

An average number of paired reads of 95,349,811 was obtained, of which 63.49%, were mapped in pairs, 10.07% were mapped in broken pairs, and 26.43% were not mapped in any region of the genome. A total of 17,154 genes were identified, distributed across all chromosomes and mitochondrial DNA. The sequences are available in the European Nucleotide Archive database under accession number PRJEB26714.

Differential expression

A total of 404 genes presented differential expression between treatments (FC ≥ 2; P-value ≤ 0.05), of which 359 corresponded to known genes, with 170 up-regulated and 189 down-regulated genes at T15, as compared to T0. These genes were used for the functional analysis. The Supplementary material (Supplementary 2) includes the detailed list of genes, FC, and P-value.

Functional analysis

Through the DAVID software, 26 significant BP were identified (P-value adjusted for the Benjamini correction ≤0.05) for all DEGs. The two processes with the highest number of genes were response to organic substance (84 genes, P-value 0.010), and cell surface receptor signaling pathway (77 genes, P-value 0.025). Other relevant processes were response to lipids (33 genes, P-value 0.039), and muscle tissue development (19 genes, P-value 0.041). For MF, three terms related to glycosaminoglycan binding (15 genes, P-value 0.0058) were recorded. As for the CC, six terms were obtained related to the extracellular region, such as extracellular matrix (32 genes, P-value 3.3 E-06) and extracellular region (115 genes, P-value 3.3 E-06). Figure 1 shows the DEGs count for the terms BP, MF, and CC. We further identified two significant KEGG pathways: focal adhesion (15 genes, P-value 0.021) and extracellular matrix (ECM)-receptor interaction (9 genes, P-value 0.045). Supplementary tables: Supplementary 3.1, Supplementary 3.2, Supplementary 3.3 and Supplementary 3.4 details the complete listing of BP, MF, CC, and KEGG_PATHWAY respectively obtained with DAVID software.

Blue represents the biological processes, red represents the molecular functions, and green represents the cellular components. The bar plot represents the gene counts within each gene ontology category. Analysis performed in DAVID v 6.8 online software.

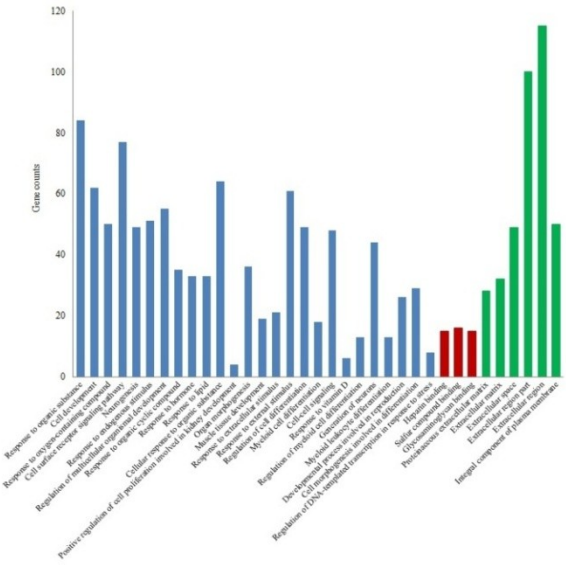

Figure 1. Classification of differentially expressed genes (DEGs) according to Gene Ontology (GO) in the *Longissimus dorsi* muscle of pigs fed with diets with different lipid contents (T0 – no added rice bran vs. T15- 15% rice bran added).

By comparing up-regulated and down-regulated genes with the ClueGo application, 26 significant groups were obtained (Supplementary 4.1). Processes and pathways assigned to clusters of up-regulated genes include focal adhesion, transport of calcium and regulation of ionic homeostasis, and protein digestion and absorption; while those for the down-regulated gene cluster are response those of hexose and skeletal muscle cell differentiation. Figure 2 shows the functional distribution of the up-regulated genes in the T15 treatment compared to T0.

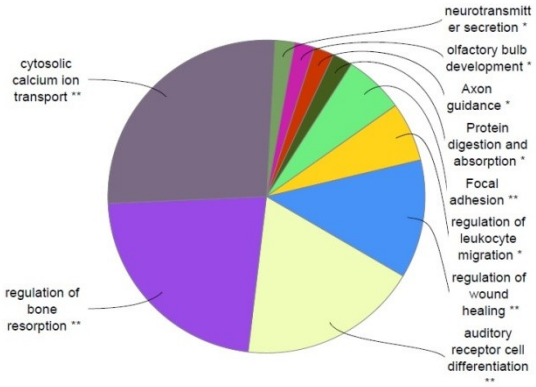

Figure 2. Summary chart showing the distribution of biological processes and pathways specific for up-regulated genes in the T15 treatment (higher lipid content) compared to T0 (control) in the *Longissimus dorsi* muscle of pigs. The name of the group is given by its most significant term. Analysis and visualization performed in ClueGo-Cytoscape.

Some processes, including regulation of lipid biosynthetic process, were not specific to any cluster and had similar numbers of up and down-regulated genes. This latter process is part of a group of processes where the rest of the terms were specific to the cluster of down-regulated genes in T15 and related to carbohydrate and steroid metabolism (Group 25, Supplementary 4.1).

Common processes resulting from functional analysis include: 1) lipid response and carbohydrate metabolism (Table 1); 2) muscle tissue development and cellular differentiation of skeletal muscle (Table 2), and 3) ECM-receptor interaction and focal adhesion (Table 3). Carbohydrate metabolism was a process obtained exclusively with ClueGo, which merits further study (Table 1).

Table 1. Biological processes (Gene Ontology) related to lipid and carbohydrate metabolism of the differentially expressed genes in *Longissimus dorsi* muscle of pigs fed diets with different lipid contents (T0 vs. T15 = 0 versus 15% added rice bran).

Response to lipid[a]	**Genes**	**P-value**
Up-regulated in T15	*TNC, CXCL11, CXCL10, LOX, SRD5A2, SPP1, PRKCA, SELP, ABR, PDK4, GPER1, SLIT3, EPHA3, TNFRSF10C, NEDD4*	0.039
Down-regulated in T15	*MYOD1, NR6A1, FOS, TYR, NR1D1, MYC, DEFB1, ZFP36, STC2, NR4A2, PIM1, NR4A3, PCK2, JUNB, GH1, CATSPER4, DUSP1, JUN*	
Regulation of lipid biosynthetic process[b*]	**Genes**	**p-value**
Up-regulated in T15	*ABHD6, DKK3, GPER1, PDK4*	0.006
Down-regulated in T15	*CYR61, EGR1, INSIG1, NR1D1, SIK1*	
Regulation of carbohydrate biosynthetic process[b]	**Genes**	**p-value**
Up-regulated in T15	*GPER1*	0.041
Down-regulated in T15	*NR1D1, PPP1R3B, SESN2, SIK1*	
Response to hexose[b]	**Genes**	**p-value**
Up-regulated in T15	*GPER1, PRKCA, RMI1*	0.003
Down-regulated in T15	*EGR1, FGF21, FOXA2, HNF1B, LIN28A, NR1D1, PCK2, SESN2, SLC26A6, VSNL1*	
Gluconeogenesis[b]	**Genes**	**p-value**
Up-regulated in T15	----------	0.043
Down-regulated in T15	*ATF3, NR1D1, PCK2, SESN2, SIK1*	

[a]Biological processes obtained with DAVID software. This analysis was performed for all DEGs without discriminating their expression level between treatments. In the table, up and down-regulated genes are shown separately to facilitate its comprehension by the readers. The P-value of biological processes corresponds to Fisher's Exact Test (EASE score), adjusted for Benjamini correction. [b]Biological processes obtained with ClueGo-Cytoscape (up and down-regulated genes in T15 compared to T0). *As the number of up and down-regulated genes was similar, the process "regulation of lipid biosynthetic" was unspecific. This process is part of a group where the remaining terms were specific to the cluster of down-regulated genes in T15 and related to carbohydrate metabolism. The P-value of biological processes corresponds to hypergeometric test, adjusted for Benjamini correction.

Table 2. Biological processes (Gene Ontology) related to muscle tissue development and cellular differentiation of skeletal muscle of differentially expressed genes in *Longissimus dorsi* muscle of pigs fed diets with different lipid contents (T0 vs. T15 = 0 versus 15% added rice bran).

Muscle tissue development[a]	**Genes**	**P-value**
Up-regulated in T15	*EOMES, C16ORF89, GJA5, EYA2, ITGA8, DNER, HEY2, COL11A1, MYLK*	0.041
Down-regulated in T15	*EGR1, MAFF, MYOD1, PIN1, FOS, ATF3, BTG2, VEGFA, SIK1, IFRD1*	
Skeletal muscle cell differentiation[b]	**Genes**	**P-value**
Up-regulated in T15	*EOMES*	
Down-regulated in T15	*ATF3, BTG2, EGR1, FOS, MAFF, MYOD1, MYH7B*	0.005
Skeletal muscle tissue development[b]	**Genes**	**P-value**
Up-regulated in T15	*DNER, EOMES*	
Down-regulated in T15	*ATF3, BTG2, EGR1, FOS, MAFF, MYOD1, MYH7B*	0.006

[a]Biological processes obtained with DAVID software. This analysis was performed for all DEGs without discriminating their expression level between treatments. In the table, up and down-regulated genes are shown separately to facilitate its comprehension by the readers. The P-value of biological processes corresponds to Fisher's Exact Test (EASE score), adjusted for Benjamini correction). [b]Biological processes obtained with ClueGo-Cytoscape (up and down-regulated genes in T15 compared to T0). The P-value of biological processes corresponds to hypergeometric test adjusted for Benjamini correction).

Table 3. Pathways obtained in KEGG database of differentially expressed genes in *Longissimus dorsi* muscle of pigs fed diets with different lipid content (T0 vs. T15 = 0 versus 15% added rice bran).

ECM-receptor interaction	**Genes**	**P-value**[a]
Up-regulated in T15	*CHAD, COMP, ITGA8, LAMC3, SPP1, THBS4, TNC*	0.0053
Down-regulated in T15	-----	
Focal adhesión	**Genes**	**P-value**[a]
Up-regulated in T15	*CHAD, COMP, ITGA8, LAMC3, MYL5, MYLK, MYLK4, PRKCA, SPP1, THBS4, TNC*	0.003
Down-regulated in T15	*JUN, VEGFA*	

Up and down-regulated genes in T15 compared to T0. [a]P-value of biological processes to the hypergeometric test, adjusted for the Benjamini correction (Obtained in ClueGo-Cytoscape).

DISCUSSION

A total of 17,154 transcripts distributed throughout the porcine genome were identified, of which 359 with known function presented differential expression between treatments and were associated with numerous biological processes and metabolic pathways. Here, we discuss the most relevant and enriched ones in the comparison between groups: lipid and carbohydrate metabolism, muscle tissue development, focal adhesion, and ECM-receptor interaction.

Lipid and carbohydrate metabolism

In the functional analysis, the genes obtained in the process "response to lipid", obtained by DAVID software, were associated with lipopolysaccharide response, signal transduction, regulation of transcription and gene expression, and inflammatory response.

The process "regulation of lipid biosynthetic process", obtained through the ClueGo application, was unspecific and formed a group where the remaining processes were related to carbohydrate metabolism, which was down-regulated. For this reason, lipid and carbohydrate metabolism will be discussed together (Table 1).

Among the up-regulated genes in T15 related to these metabolic pathways, we find *PDK4* (pyruvate dehydrogenase kinase 4), which encodes a mitochondrial enzyme involved in the shift of energy source from glucose to fatty acids in response to physiological conditions (Araki and Motojima, 2006). Lan et al., (2009) identified a mutation in this gene associated with IMF and water content in pigs. Li et al. (2016) also found a higher expression in the Chinese Wannanhua breed, characterized by better meat quality and higher IMF when compared to Yorkshire. These authors suggest that differences in their expression pattern would contribute to phenotypic differences observed in the LD muscle between different races. Another up-regulated gene was *GPER1* (G protein-coupled estrogen receptor 1), which plays an essential role in metabolic regulation, including lipid and glucose homeostasis. Estrogen-dependent signaling through GPER helps counteract the development of obesity, being seen in knockout mice for this gene the development of obesity and increase of fat deposits (Barton and Prossnitz, 2015). We also identified the *ABHD6* gene (α/β hydrolase domain containing 6), which encodes a lipase capable of degrading different lipid substrates, as an up-regulated one. Suppression of ABHD6 in mice avoids obesity and hepatic steatosis induced by high-fat diets (Thomas et al., 2013, Zhao et al., 2016). For its role in energy homeostasis, it is considered a potential therapeutic target for diseases that alter lipid metabolism, such as obesity and type II diabetes. The up-regulation of *GPER1* and *ABHD6* may counteract the effect of the diet with higher lipid content (T15 treatment) and be related with the down-regulation of the metabolic pathways of carbohydrates that we observed.

Regarding the down-regulated genes associated with these pathways, most encode transcription factors (TF). Among these, we find *MYC* (myc proto-oncogene protein), which is involved in adipocyte proliferation, lipid metabolism, and fatty acid transport. Ayuso et al. (2016) point out that it is a potential TF for regulation of fat gain in young pigs. Other down-regulated genes were *JUNB* (JunB proto-oncogene), *JUN* (jun proto-oncogene) and *FOS* (proto-oncogene c-Fos), which encode members of the AP-1 family (activating protein 1), and take part in the regulation of several cellular processes including proliferation, differentiation, apoptosis, and oncogenesis (Chinenov and Kerppola, 2001). Puig-Oliveras et al., (2016) found six SNPs in *FOS* associated with IMF and fatty acid (FA) composition, being an important candidate gene for the study of these complex characteristics affecting meat quality. Other down-regulated genes were *NR4A2* and *NR4A3* (nuclear receptor subfamily 4 group A member 2 and member 3, respectively), which encode nuclear receptors expressed in tissues with high energy demand such as skeletal muscle. They respond directly to signaling molecules, including fatty acids, and their differential expression in human adipose tissue would affect metabolic processes such as lipolysis, lipogenesis, glucose transport, and energy expenditure (Veum et al., 2012).

The fact that pathways associated with lipid metabolism are nonspecific agrees with the lack of differences in IMF and FA profiles. When analyzing the influence of dietary oils on the fatty acid composition of the *longissimus* muscle, Teye et al., (2006) also did not found differences in IMF. These authors found that one of the diets used, which included soya bean, was high in PUFA (especially 18:2n-6); leading to a significant increase in the

concentrations of these fatty acids in the muscle. Nuernberg et al., (2005), when comparing two diets, one composed of 5% olive oil (rich in monounsaturated fatty acids) and the other with 5% linseed oil (rich in polyunsaturated fatty acids), found that in the latter case linolenic acid was significantly increased in muscle lipids.

This lack of differences in our research could be due either to the short time the animals were subjected to the treatments, or to the fact that the difference in lipid content between the diets was not large enough. It could also be due to the number of individuals analyzed, since Montenegro et al. (2018), analyzing these same animals within a larger population, found differences in PUFA and linoleic acid values. However, it could be expected that in those animals fed with the diet with higher lipid content there has been an increase in the availability of lipids at the muscle level. This would explain the identification of up-regulated genes that are negative regulators of lipid biosynthesis and have lipase activity; as well of down-regulated genes related to carbohydrate metabolism. Puig-Oliveras et al. (2014) reported similar results in LD muscle of pigs with different fatty acid composition, where a higher PUFA content could reduce consumption of fatty acids and glucose and result in an inhibition of lipogenesis. Pascual et al. (2007) state that under isocaloric diets, the addition of dietary lipids may induce a decrease in endogenous lipid synthesis in parallel with reduced carbohydrate utilization, which is the main substrate for lipogenesis. In our case, up-regulated genes indicating a decrease in lipid utilization, together with the decrease in pathways and genes linked to carbohydrate metabolism, would indicate that the higher lipid content of T15 affected to some extent these metabolic pathways. The functions of genes related to these pathways, together with the evidence relating them to different diseases, make necessary to deepen into their knowledge, mainly if we consider that pig is a model species for the study of human diseases.

Muscle tissue development

Most of the DEGs identified as related to skeletal muscle development were down-regulated (Table 2). Among them, we found important regulators such as *FOS*, *ATF3* (activating transcription factor 3) and *MAFF* (MAF bZIP transcription factor F). FOS is a member of the aforementioned AP-1 family and, in addition to influencing the accumulation of IMF, is an important regulator of muscle differentiation and metabolism (Ayuso et al., 2016; Hou et al., 2016). Hou et al. (2016) reported a lower level of expression in Landrace, compared to Chinese races, suggesting that this differential expression could influence the regulation of skeletal muscle growth. In the case of ATF3 and MAFF, they are TFs that interact with members of the AP-1 family. ATF3 is a cAMP-dependent TF related to positive regulation of cell proliferation and differentiation of skeletal muscle cells. Wang et al. (2015) reported an increase in its expression same in the Chinese creole races Diannan Small-ear and Tibetan, suggesting that it could be a candidate gene for inhibition of muscular growth in these races. Keller et al. (2011) reported a down-regulation of *FOS* and *ATF3* in pigs fed with a diet supplemented with L-carnitine, and suggest that this decrease would have an anti-apoptotic effect on skeletal muscle of pigs, mainly due to the inhibition of pro-apoptotic signaling pathways. The effect of apoptosis has been demonstrated on the loss of muscle mass observed during aging and in certain pathologies, such as muscular dystrophy and neuromuscular diseases (Siu and Alway, 2009), whereby down-regulation of these genes could have a favorable effect, although there is no information on this. *MAFF* is

also related to skeletal muscle differentiation. Studying the skeletal muscle of Duroc pigs with different lipid profiles, Cardoso et al. (2017) also found a differential expression of this gene, which is sub expressed in the group with higher PUFA content. Although we did not found significant differences in the lipid profile, PUFA content was higher in those animals subject to a treatment with higher lipid content; hence, our results regarding this gene could be in agreement with those reported by Cardoso et al. (2017).

According to the stage of growth of the animals analyzed (77 days at the end of the experiment), it would be expected the expression of pathways linked to muscle growth in both groups. However, the down-regulation observed in those animals fed with the diet with higher lipid content may suggest an effect of the treatment over these pathways. Down-regulated genes encoding TFs, due to their incidence on different pathways and processes, are important candidates that could mediate the response of the organism to the difference in lipid content of the treatments used in this study.

ECM-receptor interaction and focal adhesion

These pathways were up-regulated in T15 (Table 3). The ECM consists of a complex assembly of proteins and polysaccharides that play an important role in the morphogenesis of organs and tissues, and in the maintenance of cellular and tissue structures and functions. Specific interactions between cells and ECM regulate cellular activities such as adhesion, migration, differentiation, proliferation, and apoptosis. Focal adhesions are specialized adhesive structures between cells and ECM, mediated primarily by integrin family receptors, constituting important sites for signal transduction (van der Flier and Sonnenberg, 2001; Clause and Barker, 2013). ECM affects meat quality as it can affect IMF deposition, connective tissue content and muscle development. As for muscle development, the interactions between ECM and muscle cells play a central role in regulating differentiation and proliferation of these cells (Velleman, 2012). In the ECM, the mesenchymal stem cells (MSCs) are located, from which myogenic cells are derived, as well as the satellite cells that are indispensable for postnatal muscle growth. The extracellular environment regulates the proliferation and differentiation of MSCs and satellite cells. If MSC cells progress through the adipogenic differentiation process, the IMF content will be affected; if they progress through the fibrogenic differentiation process, this will lead to the synthesis of collagen and an increase in the content of connective tissue; and if they progress through the myogenic process, it will lead to an increase in muscle mass (Du and Carlin, 2012; Óvilo et al., 2014).

Among the up-regulated genes that are part of these pathways, we found *ITGA8* (integrin subunit alpha 8), *COL11A1* and *COL11A2* (collagen type XI alpha 1 chain and alpha 2 chain, respectively). Integrins interact with a large number of proteins, and the signaling events that involve them lead to changes in cell morphology and mobility, as well as in gene expression (van der Flier and Sonnenberg, 2001; Wehrle-Haller, 2012). It has been proposed that they would intervene in angiogenesis regulation, which could in some way affect adipogenesis due to the close relationship between the two processes (Óvilo et al., 2014).

Collagen molecules are major components of ECM, and their intramuscular content contributes to meat hardness, affecting its texture and being related to the growth rate. In

turn, collagen development is negatively related to the development of adipocytes in ECM (Óvilo et al., 2014).

Among the up-regulated genes in T15, we also found *TNC*, which encodes the tenascin C protein. This ECM protein is part of a protein family that modulates cell adhesion and response to growth factors (Chiovaro et al., 2015). The *TNC* gene has been reported as a potential candidate gene for meat quality traits in pigs (Kayan et al., 2011).

These pathways have been reported as enriched in the LD muscle of pigs, with different composition in intramuscular fat (Cánovas et al., 2010; Pena et al., 2013). Óvilo et al., 2014 found higher expression in Duroc x Iberian crossbred pigs and highlight the importance of ECM in the composition and organization of muscle tissue, as well as in the regulation of IMF deposition.

In our case, the higher expression of ECM pathways and focal adhesion in those animals fed with the diet with higher lipid content, suggests an effect of the treatment, which may affect characteristics associated with meat quality, as well as the transduction of signals and the signaling of events.

CONCLUSIONS

Through RNAseq, we identified 359 genes with differential expression between groups, 170 up-regulated, and 189 down-regulated for treatment with higher lipid content when compared to the control diet. Treatment effect can be observed through the activity of these genes in important metabolic pathways, some of which affect meat quality. Among these pathways, those associated with lipid metabolism were not affected, coinciding with the similarity of IMF and fat content. We observed an effect on carbohydrate metabolism, with a lower expression of the genes involved in these pathways in the treatment with higher lipid content. Other important pathways affected were muscle tissue development and ECM-receptor interaction. As for muscle tissue development, we found a lower expression in the treatment with higher lipid content, while the opposite occurred for the ECM pathway. The results of the differential expression of the genes involved in these pathways, which was affect by the lipid content of the diet, provides information that may be useful for future research related to meat quality, also providing information that may be valuable in the study of human diseases, mainly those related to energy metabolism and muscle development.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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