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Transcriptome analysis of the Iberian pig

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Abstract. Iberian pig meat is a remarkable constituent of the healthy Mediterranean diet. The organoleptic and nutritional properties of Iberian pig products are influenced by both genetic and environmental factors including the animal's diet. To investigate the potential impact of genotype and diet on gene expression eventually controlling taste and texture of the meat, we performed an in-depth transcriptomics analysis of the Iberian versus Duroc pig muscle from differentially fed animals. Muscle tissue samples were frozen in liquid nitrogen immediately after sacrificing the animals and stored at –80 ºC. Total RNA was extracted, retrotranscribed to cDNA and subjected to SuperTag Digital Gene Expression (ST-DGE) functional genomics analysis. The generated sequences were then counted and annotated to entries in public databases to assign potential functions to the expressed genes. To this end we identified significant breed-specific as well as diet-specific expression profiles of known as well as from hitherto unknown genes, involved in metabolic pathways related to the quality of Iberian meat products. These results demonstrate the power of genomics in general and transcriptomics in particular to identify differentially expressed profiles and putative candidate genes for quality control, certification and traceability, helping breeders and farmers to produce both animals and derived products (like ham) with higher nutritional value and improved organoleptic properties, to further enhance the healthy Mediterranean diet.

Keywords. Genome – Functional genomics – mRNA – Bioinformatics.

L'analyse du transcriptome du porc Ibérique

Résumé. *La viande de porc Ibérique est un constituant remarquable de la saine alimentation méditerranéenne. Les propriétés organoleptiques et nutritionnelles des produits du porc Ibérique sont influencées par des facteurs génétiques et environnementaux, y compris l'alimentation de l'animal. Pour étudier l'impact potentiel du génotype et du régime alimentaire sur l'expression génique du goût et éventuellement le contrôle et la texture de la viande, nous avons effectué une analyse en profondeur du transcriptome du muscle du porc Ibérique par rapport aux porcs Duroc à partir d'animaux nourris différemment. Des échantillons de tissus musculaires ont été congelés dans l'azote liquide immédiatement après l'abattage des animaux et conservés à -80º C. L'ARN total a été extrait, rétrotranscrit en ADNc et soumis à une analyse génomique fonctionnelle pour l'expression des gènes moyens selon la technique SuperTag (ST-DGE). Les séquences ont alors été comptées et annotées pour leur entrée dans des bases de données publiques pour assigner des fonctions potentielles aux gènes exprimés. À cette fin, nous avons identifié des profils d'expression significatifs spécifiques de la race ainsi que de l'alimentation, pour des gènes connus ainsi qu'inconnus jusque-là, impliqués dans les voies métaboliques liées à la qualité des produits de la viande du porc Ibérique. Ces résultats démontrent la puissance de la génomique et de la transcriptomique en général, et en particulier pour l'identification des profils différentiellement exprimés et des gènes candidats putatifs pour le contrôle, la certification de la qualité et la traçabilité, afin d'aider les éleveurs et les agriculteurs à produire des animaux et des produits dérivés (comme le jambon) ayant une valeur nutritionnelle plus élevée et de meilleures propriétés organoleptiques, pour améliorer encore le sain régime méditerranéen.*

Mots-clés. *Génome - Génomique fonctionnelle – ARNm – Bioinformatique.*

I – Introduction

The quality is an important parameter of any food product. This is particularly relevant for products with a Protected Designation of Origin (PDO) certification issued by the European Union (EU). Such label corresponds to food products that are specific of a particular region, conveying a particular quality or characteristic which is peculiar of such designated area. The PDO certification requires the food traceability from the origin to the consumer. Likewise, the National Quality Standard (NQS; "Norma de Calidad") for the Iberian pig products (ham, shoulder blade and loin) in Spain regulates the labeling of such food ("Real Decreto" 1469/2007, of 2nd November). Different approaches have been developed and deployed for food certification and traceability, being particularly efficient and convenient the ones based on DNA molecular markers. Yet, the development of such markers requires the prior knowledge of the DNA sequences differentiating the products to be identified and tracked down.

On the other hand, consumers are demanding with increasing emphasis not only quality-certified food products, but also –and specially– healthy products. The Iberian pig products are remarkable constituents of the healthy Mediterranean diet, which has been included in the United Nations Educational, Scientific and Cultural Organization (UNESCO) list of "Intangible Cultural Heritage of Humanity" since 18th November 2010. The healthy implications of the Iberian pig products have not been completely elucidated, but some studies have shown that their balanced compositions of unsaturated lipids protect against cardiovascular diseases (CVD), effectively reducing the plasma levels of Low Density Lipoprotein (LDL) cholesterol, total cholesterol, triglycerides and fibrinogen (Rebollo *et al.*, 1998; Martín *et al.*, 2009; Jiménez-Colmenero *et al.*, 2010).

Remarkably, the fat composition of the Acorn-Fed Iberian Ham (AFIH) has a surprisingly high content of oleic acid (monounsaturated lipid) for a product of animal origin (50 to 60% of the total fat), depending on the tissue considered. Thus, on the coccyx fat it reaches up to 57% (about 60% monounsaturated lipids), whereas in the intramuscular fat the oleic acid represents 45 to 50% and the saturated fats about 40%. The high oleic acid content of the Iberian ham resembles the one of the olive oil, which ranges from 55.0 to 83.0% of oleic acid content depending on cultivar. In this respect, it should be emphasized that the olive oil has been granted the label of "Qualified Health Claim" by the Food and Drug Administration (FDA) of the United States of America, due to its protective effect against CVD.

The organoleptic, nutritional and healthy properties of the Iberian pig products are influenced by both genetic and environmental factors including the animal's diet (eg., the acorn feeding, as previously indicated; the acorn has a similar lipid composition to the olive oil itself). To investigate the potential impact of genotype and diet on gene expression eventually controlling taste, texture and composition of the meat, we have performed an in-depth transcriptomics analysis of the Iberian *versus* Duroc pig muscle from ham of differentially fed animals. The final goals of this research are both to unravel the genomic, transcriptomic and metabolomic pathways that set apart the Iberian pig from other breeds, as well as to identify differentially expressed genes that can be used as tools for the certification and traceability of Iberian pig products.

Explanatory note: The purpose of this work is not to carry out a statistical analysis of many samples because: i) this kind of genomic experiments use a different methodological approach in the literature; and ii) the prohibitive cost of such statistical approach on genome-wide experiments, not being cost-effective. On the contrary, the purpose of this work is to carry out a genome-wide transcriptomic analysis in order to discover candidate genes that are highly differentially expressed (upregulated or downregulated) between the breeds and feedstuffs used. Such candidate genes are now being subjected to other analysis (validation using many animals from different herds), but such work is out of the scope of the present report. The readers interested on the methodology used in genomic studies are directed to the abundant bibliography on such matter for further details, as previously indicated.

II – Materials and methods

Female Iberian pigs from the most abundant lineage (Retinto) were selected from the same brood and fatten up for 70 days with either strict “montanera” (acorn-based with grass), standard cereal-based feedstuff without acorn (as well as female Duroc, used as control) or standard cereal-based feedstuff enriched with olein (oleic-acid-rich feedstuff). The standard feedstuff contained (mg/kg): barley (14.75 x 10^4), wheat (50.00 x 10^4), corn (21.00 x 10^4), soybean meal (1.20 x 10^4), lard (1.10 x 10^4), calcium carbonate (8.00 x 10^3), calcium phosphate (1.15 x10^4), sodium chloride (4.00 x 10^3) and corrector (5.25 x 10^3). The olein-supplemented feedstuff contained (mg/kg): barley (47.50 x 10^4), wheat (15.00 x 10^4), bran (15.50 x 10^4), soybean meal (8.50 x 10^4), pulped beet (5.75 x 10^4), high-oleic sunflower meal (5.75 x 10^4), calcium carbonate (9.50 x 10^3), calcium phosphate (7.00 x 10^3), sodium chloride (4.00 x 10^3), corrector (5.00 x 10^3) and vitamin E mix (250 ppm). The Table 1 summarizes the three feedstuffs used.

Table 1. Feedstuff composition

Composition (%)*	“Montanera”		Standard	Olein
	Acorn	Grass		
DM	54.70	10.60	85.62	89.91
DM ash	2.11	13.54	3.89	4.56
DM crude protein	7.97	21.85	16.78	16.94
DM crude fiber	1.82	20.00	3.28	5.67
DM fat	7.06	3.07	3.55	8.54
DM NFE	80.50	46.60	72.57	64.35
SFA	12.12	21.75	25.15	11.15
MUFA	64.65	7.34	32.08	67.50
PUFA	21.60	70.92	42.68	21.52

(*): DM: Dry Matter; NFE: Nitrogen-Free Extracts; SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids.

The pigs were transported to the slaughterhouse the day before slaughtering, trying to minimize the stress of the animals. Carbon dioxide was used for stunning just before bleeding, according to the specifications outlined in the European Union legislation. The Iberian pigs were slaughtered after 14 months old (150 to 170 kg); the Duroc pig grows faster and thus was slaughtered after 9 months old (157 kg).

The ham muscle tissue samples (*biceps femoris*; femoral biceps) were frozen in liquid nitrogen immediately after sacrificing the animals, and stored at –80 ºC until needed. The total RNA was isolated from samples using Trizol from Life Technologies (Carlsbad, CA, USA). In short, the tissue was ground under liquid nitrogen with a pestle and mortar. A small volume of ground tissue was dissolved in 1 ml Trizol and homogenized. The RNA was isolated after the addition of 1/5 volume of chloroform. Subsequently, the RNA was precipitated with isopropanol, washed and dissolved in diethylpyrocarbonate-treated water. The RNA concentration was determined using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). The RNA quality (integrity) was checked by agarose gel electrophoresis. The RNA was retrotranscribed to cDNA with reverse transcriptase and subjected to SuperTag Digital Gene Expression (ST-DGE) functional genomics analysis (Anisimov, 2008; Datson, 2008; Matsumura *et al.*, 2008a,b, 2010; Wang, 2008; Zaretzki *et al.*, 2010). The generated sequences were then counted and annotated to entries in public databases to assign potential functions to the expressed genes.

III – Results and discussion

As previously indicated, the main purpose of this work is not to carry out a statistical study with many animals, but to use the currently validated genomic methodological approaches to carry out a genome-wide transcriptomics analysis. The usefulness of such strategy to identify differentially expressed genes and thus putative candidate genes for further applications is widely supported in the literature. More than a million Expressed Sequence Tags (EST) have been isolated and sequenced, including differentially expressed ones between breeds and feeds. Thus, we have identified breed-specific as well as diet-specific expression profiles of known as well as from hitherto unknown genes, some of them being involved in metabolic pathways related to the characteristics of Iberian meat products.

The transcriptomic profiling of Iberian pigs fed with either acorn, standard feedstuff or olein were compared with the transcriptomic profiling of Duroc pigs fed with standard feedstuff. A total of 15,814 to 18,952 expressed tags were compared for each pair of breeds and feeding, generating gene expression fold changes from +14.58 to +18.09 on one side and –13.71 to –16.58 on the other side. This represents a remarkable wide range of variation.

The analysis of such data has allowed the identification of differentially expressed genes (eg., involved in lipid and peptide metabolism and transport), besides genes that are currently unknown. The expression of genes related with lipid biosynthesis was higher on Iberian *vs* Duroc pigs. Likewise, for acorn-fed versus standard-fed Iberian pigs. These results indicate that both the genotype as well as the diet determine the higher lipogenic gene expression in the acorn-fed Iberian pig.

These results demonstrate the power of genomics in general, and transcriptomics in particular, to identify differentially expressed genes between pig breeds and feeding conditions. The data generated will contribute to a better understanding of the pig transcriptomics and metabolomics pathways. These results are also being exploited and validated analyzing many individuals from different herds (data not shown, corresponding to future publications) to design DNA molecular markers for quality control, certification and traceability. These developments will help breeders and farmers to produce both animals and derived products (like ham) with higher nutritional value and improved organoleptic properties, to further enhance the healthy Mediterranean diet. Likewise, they can contribute to increase the consumer confidence for products with higher quality and price, which can further assist the promotion and consolidation of demanding markets (Japan, USA, etc).

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