UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

Aplicación de nuevas tecnologías -ómicas y de fenotipado en programas de selección para calidad de carne en cerdos Ibéricos de montanera

MIGUEL ÁNGEL FERNÁNDEZ BARROSO

PROGRAMA DE DOCTORADO EN CIENCIA DE LOS ALIMENTOS

La conformidad de los directores de la tesis consta en el original en papel de esta Tesis doctoral:

María Muñoz Muñoz

Juan María García Casco

2021

UNIVERSIDAD DE EXTREMADURA

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

INIA
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria

TESIS DOCTORAL

Aplicación de nuevas tecnologías -ómicas y de fenotipado en programas de selección para calidad de carne en cerdos Ibéricos de montanera

Application of new -omic and phenotyping technologies for meat quality traits in Iberian montanera pigs breeding programs

MIGUEL ÁNGEL FERNÁNDEZ BARROSO

DIRECTORES: MARÍA MUÑOZ MUÑOZ Y JUAN MARÍA GARCÍA CASCO

CENTRO NACIONAL DE I+D DEL CERDO IBÉRICO. DPTO MEJORA GENÉTICA ANIMAL (CSIC-INIA)

PROGRAMA DE DOCTORADO EN CIENCIA DE LOS ALIMENTOS

2021

Los doctores María Muñoz Muñoz y Juan María García Casco, investigadores del Centro Nacional de I+D del Cerdo Ibérico y del departamento de Mejora Genética Animal del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (CSIC-INIA),

CERTIFICAN:

Que la Tesis Doctoral titulada "**Aplicación de nuevas tecnologías -ómicas y de fenotipado en programas de selección para calidad de carne en cerdos Ibéricos de montanera**" presentada por Miguel Ángel Fernández Barroso para optar al grado de Doctor, ha sido realizada bajo su dirección, cumple las condiciones exigidas para obtener dicho título y autoriza su presentación para que sea juzgada por la comisión correspondiente.

Y para que así conste, firman en Badajoz, a 11 de junio de 2021.

María Muñoz Muñoz Juan María García Casco

AGRADECIMIENTOS

AGRADECIMIENTOS

En primer lugar, me gustaría agradecer a mis directores María Muñoz y Juan García por la confianza depositada durante el proceso de elaboración de la tesis doctoral, la cual que se ve reflejada en este trabajo. También por estar siempre disponibles, a pesar de las actuales condiciones de incertidumbre en la que a nos ha tocado desarrollar esta tarea. Por las enseñanzas, las revisiones, por todo el aprendizaje y la paciencia que han tenido. Por ofrecerme la oportunidad de participar en congresos científicos, gracias a los cuales he podido crecer profesionalmente, pero también personalmente, conociendo diversos lugares y culturas.

A Juan Florencio Tejeda, por estar siempre disponible como tutor, por orientarme y ayudarme. A Elena González (directora por un tiempo) por su apoyo y poder aprender de todos sus conocimientos.

A mis compañeros de Zafra del Centro I+D del cerdo Ibérico, Carmen, José y Patricia, por el buen ambiente de trabajo que hemos tenido, por echar una mano constantemente, por lo fácil que hacen el día a día, trabajando todos coordinados en equipo, incluyendo aquí a Juan y María, especialmente esos largos días de viajes matadero, de viajes a campo y de preparación de muestras en el laboratorio.

A Luis Silió y Carmen Rodríguez, por toda la ayuda con las revisiones para los artículos y compartir todos sus conocimientos y experiencia. Ambos son parte fundamental del equipo fundador de investigadores que idearon hace unas décadas este proyecto de programa de mejora en cerdo Ibérico.

A todos mis compañeros del departamento de mejora genética en Madrid, Yolanda, Adrián, Fabián, Rita y tantos otros, por toda la ayuda y disponibilidad. Por el buen ambiente durante las semanas de estancia allí.

A todo el equipo de Sánchez Romero Carvajal (SRC). A José M. Pariente por la gran gestión y organización. A Fernando Gómez, Fernando Sánchez, Gema, Luisa y Alberto, por toda la asistencia, su dedicación e involucración en el proyecto. También a todas las personas que trabajan en el campo y en la industria alimentaria, que de algún u otro modo, han contribuido con su esfuerzo en este estudio.

A Ana Mercader de la UAB por su disponibilidad y asistencia en el genotipado del panel de caracteres calidad.

A todo el grupo de la Università degli Studi di Firenze. A Riccardo Bozzi, Silvia Parrini, Carolina Pugliese, Alessandro y al resto de compañeros. Por brindarme la oportunidad de realizar una estancia internacional con ellos, por ayudarme en todo y acogerme como uno más.

A Ana y Jose. Por la amistad verdadera, por saber escuchar y por sus consejos. Por el viaje a Monfragüe, por las conversaciones sobre buitres negros.

A mis padres y mi hermana. Por todo el apoyo incondicional, el cariño, por inculcarme siempre los valores del trabajo y esfuerzo.

A Laura, por comprenderme, por el cariño, paciencia y por confiar en que podía lograrlo.

ÍNDICE

Índice

Índice de Tablas

Índice de Figuras

RESUMEN

El cerdo Ibérico pertenece al grupo de razas autóctonas europeas caracterizadas por la ausencia de una selección intensiva orientada a la producción. Además, a diferencia de la mayoría de las razas porcinas comerciales europeas, la ausencia de introgresión procedente de razas asiáticas o de otras razas magras europeas hace que la raza Ibérica sea genéticamente singular. El cerdo Ibérico se caracteriza fenotípicamente por tener un potencial adipogénico elevado, resultado de su alta capacidad de ingesta. También se caracteriza por tener una excelente calidad organoléptica de la carne y productos cárnicos. Estas características están determinadas por la combinación de su genética y el sistema tradicional de producción, basado en un periodo final de engorde al aire libre conocido como montanera, en el cual el cerdo se alimenta de bellotas, pastos y raíces ad libitum. Este periodo final de engorde favorece la infiltración de grasa en los tejidos musculares y la presencia de un mayor contenido de ácidos grasos monoinsaturados, especialmente de oleico. Actualmente, existen cinco variedades o estirpes reconocidas oficialmente en el Libro Genealógico: Torbiscal, Lampiño, Manchado de Jabugo, Retinto y Entrepelado. La presente tesis se ha realizado con datos procedentes de cerdos de la variedad Retinto.

La calidad de la carne es un factor relevante reconocido por la industria de la cual se pueden considerar cuatro acepciones: calidad tecnológica, calidad organoléptica, calidad nutricional y calidad higiénico-sanitaria. Tradicionalmente los programas de selección en porcino se han centrado en mejorar con éxito caracteres productivos, como son la tasa de crecimiento o el espesor de la grasa dorsal. Sin embargo, no existen demasiados programas de mejora implementados en cerdo Ibérico y mucho menos los que tienen como objetivo de selección los caracteres de calidad de carne. Por lo tanto, el estudio de un programa de mejora para cerdos Ibéricos centrado en los caracteres de calidad de carne está totalmente justificado.

El objetivo general de la presente tesis ha sido el uso de la información molecular obtenida de la aplicación de técnicas ómicas (genómica y transcriptómica) en un programa de mejora de cerdos Ibéricos alimentados en el sistema de montanera, así como la aplicación de nuevas tecnologías de fenotipado, orientado en ambos casos hacia los caracteres de calidad de carne.

Para ello se caracterizó fenotípica y genéticamente una población de cerdos de una línea Ibérica cerrada a la entrada de reproductores externos desde hace 15 años. Se midieron los siguientes caracteres de calidad de carne en muestras de músculo longissimus dorsi: pérdidas de agua por descongelado, cocinado y por fuerza centrífuga (atributos de capacidad de retención de agua); color instrumental Minolta de luminosidad (L*), rojo (a*) y amarillo (b*) y contenido en mioglobina (atributos de color); medida de la resistencia al corte y fuerza de compresión (atributos de textura). A partir del registro de datos de estos caracteres en la población de referencia, se realizaron cuatro experimentos.

El objetivo del primer experimento fue la caracterización de la población para este conjunto de caracteres de calidad de carne, la estimación de los parámetros genéticos (heredabilidad y correlaciones genéticas) y la evaluación de los efectos potenciales de un panel de SNPs mapeados en genes candidatos. En general, los valores de las estimas de heredabilidad fueron moderados para la mayoría de los caracteres, siendo la heredabilidad para las pérdidas de agua por cocinado (0.43) la más elevada. Respecto a las correlaciones genéticas, las más altas se observaron entre los caracteres de pérdidas de agua (0.93 a 0.96), así como entre estas pérdidas de agua y la resistencia al corte en carne cocinada (0.71 a 0.91) y entre el contenido en mioglobina y el parámetro de color a* (0.94). El análisis de asociación reveló 19 SNPs asociados significativamente con los distintos caracteres. Se observaron asociaciones consistentes entre SNPs localizados en los genes *PRKAG3*, *MYH3*, *CASP3* y *CTSL* con las pérdidas de agua. Y también entre SNPs de *CAPN1* y *CASP3* con la resistencia al corte. Dos SNPs de *CASP9* mostraron efectos significativos para el contenido en mioglobina. Además, los SNPs mapeados en el gen *PRKAG3* mostraron los efectos mayores en los caracteres de color Minolta.

El objetivo del segundo experimento fue evaluar la precisión de la metodología NIRS para cuantificar un conjunto de 10 caracteres de calidad de carne en muestras enteras y trituradas, usando la región completa del infrarrojo cercano en lomos procedentes de la misma línea de cerdos Ibéricos. La técnica NIRS podría ofrecer la oportunidad de cuantificar o clasificar un elevado número de muestras simultáneamente, para el conjunto de caracteres de calidad, con una única lectura, por

lo que sería una técnica rápida y limpia. Los resultados de estos análisis se evaluaron en función del coeficiente de determinación (R^2), la media del error de la raíz cuadrada (RMSE), la desviación de predicción residual (RPD) y la ratio del rango de error (RER). Los valores más elevados para el coeficiente de determinación en validación externa (R^2p) para muestras enteras fueron para los atributos de dureza y color rojo (a*) y amarillo (b*) ($0.7<R^2p<0.8$). Mientras que, para muestras trituradas, los de luminosidad (L*), contenido en mioglobina y los parámetros de textura obtuvieron valores de $R^2p>0.7$. Los modelos estadísticos desarrollados sugieren la habilidad de la técnica NIRS para la cuantificación del carácter de resistencia al corte en muestras trituradas. Además, es posible una clasificación aproximada para los caracteres de cohesividad en muestras trituradas, así como para los parámetros de color (L*, a*, b*) y dureza en muestras enteras.

El objetivo del tercer y el cuarto experimento consistió en analizar mediante la técnica RNA-seq los genes que estaban diferencialmente expresados entre animales extremos para dos caracteres de calidad diferentes. Se seleccionaron dos grupos de cerdos Ibéricos con valores mejorantes divergentes para terneza (experimento 3) y contenido en mioglobina (experimento 4) para la caracterización del transcriptoma en el músculo longissimus dorsi. Se estudió y se intentó comprender la regulación de los procesos biológicos, rutas metabólicas y funciones en los que estaban implicados los genes diferencialmente expresados y que pudieran afectar a la variabilidad de la expresión fenotípica en ambos caracteres. Respecto a los análisis de los grupos divergentes para terneza, el análisis funcional mostró un enriquecimiento de genes expresados diferencialmente en procesos biológicos relacionados con proteólisis (*CTSC*, *RHOD*, *MYH8*, *GADD45B*, *ACTC1*, *CASQ2*, *CHRNA9*), desarrollo del tejido muscular esquelético (*ANKRD1*, *DMD*, *MSTN* y *FOS*), metabolismo lipídico (*FABP3* y *PPARGC1A*) y metabolismo del colágeno (*COL14A1*). El análisis de expresión diferencial entre grupos divergentes para el contenido en mioglobina mostró un enriquecimiento de genes expresados diferencialmente involucrados en procesos relacionados con trasporte de oxígeno (*HBA1*), metabolismo lipídico (*ECH1*, *PLA2G10* y *PLD2*), inflamación (*CHST1*, *CD209* y *PLA2G10*) y sistema inmune (*CD209*, *MX2*, *LGALS3*, *LGALS9, SLA1* y *SLA7*).

Los resultados obtenidos en esta tesis han mostrado que los datos de genotipado de *PRKAG3_rs319678464G > C*, *PRKAG3_rs330427832C > T* SNPs y *CAPN1_rs81358667G > A* probablemente podrán ser usados en la selección de verracos para la mejora de las pérdidas de agua (*PRKAG3*) y terneza (*CAPN1*). Además, en el análisis del transcriptoma no sólo se han encontrado dos conjuntos de genes candidatos, uno para terneza y otro para contenido en mioglobina, sino que también han proporcionado información sobre los procesos biológicos y redes génicas subyacentes para ambos caracteres. Por último, los resultados con la metodología NIRS la señalan como una herramienta prometedora para la clasificación de muestras intactas en tres categorías, de acuerdo con el parámetro de terneza, además de como un posible sustituto para la técnica de textura Warner-Bratzler. En términos generales, los resultados de esta tesis serán muy útiles en la producción de cerdo Ibérico y abre nuevos caminos para el estudio y aplicación de la información molecular procedente de las tecnologías ómicas en esta raza.

SUMMARY

The Iberian pig breed belongs to the group of autochthonous European pig breeds characterized by the absence of a systematic an intensive productive selection. Moreover, unlike most of the commercial European pig breeds, the Iberian have a genetic uniqueness due to the lack of introgression from Asian breeds or other European lean breeds. The Iberian pig breed is phenotypically characterised by a high adipogenic potential, as result of its high intake capacity, as well as the great organoleptic quality of its meat and its cured products. These features are determined by the combination of their genetic characteristics and its traditional production system, based on an open-air free-range finish-fattening period named *montanera*, with ad libitum intake of acorns, pastures and roots. This final fattening period reinforces their intramuscular fat content, with a high proportion of monounsaturated fatty acids, especially oleic fatty acid. Currently, there are five varieties or strains officially recognised in the Herd-book: Torbiscal, Lampiño, Manchado de Jabugo, Retinto and Entrepelado. The present thesis has been carried out using the data set collected from a population belonging to the most abundant of them, the Retinto variety.

Meat quality is a well-known relevant factor for the industry and includes four principal meanings: technological, organoleptic, nutritional and health-hygienic quality. Traditionally, porcine breeding programs have been focused on successfully improving production traits such as growth rate or backfat thickness. However, there are not many breeding programmes implemented in the Iberian pig breed so far, much less focused on meat quality traits as a selection goal. Therefore, a study of an Iberian pig breeding program that pays attention on meat quality traits is fully justified.

The main objective of the current thesis has been the use of the molecular information obtained from the application of -omics techniques (genomic and transcriptomic) in a breeding program of Iberian pigs fattened in the *montanera* open-air free-range system, focused on meat quality traits and using new techniques of phenotyping.

To meet this objective, the following meat quality traits were determined in a Iberian pig line genetically closed since 15 years: thawing, cooking and centrifuge force water losses (as water holding capacity features); instrumental Minolta colour of lightness (L*), redness (a*) and yellowness (b*) and myoglobin content (as colour

features); tenderness (shear force) and maximum compression force by texturometer (as texture features). From the recording data of these traits in the reference population, four experiments were carried out.

The aim of the first experiment was to characterize the population for the set of meat quality cited traits in longissimus dorsi muscle samples, to estimate their genetic parameters (heritabilities and genetic correlations) and to evaluate the potential effects of a SNPs panel mapping on candidate genes through association analyses. In general, estimated heritability values were moderate for most of the traits, being the highest one for cooking loss (0.43). Strong genetic correlations were observed between water holding capacity traits (0.93 to 0.96), also between shear force on cooked meat and water holding capacity (0.71 to 0.91) and between myoglobin content and a* (0.94). The association analyses revealed 19 SNPs significantly associated with different traits. Consistent and strong effects were observed between SNPs of *PRKAG3*, *MYH3*, *CASP3* and *CTSL* and water losses. Also, between SNPs of *CAPN1* and *CASP3* and shear force. Two *CASP9* SNPs showed significant effects on myoglobin content. In addition, the SNPs mapping on *PRKAG3* showed the highest effects on Minolta colour traits.

The aim of the second experiment was to assess the accuracy of the NIRS methodology to quantify a set of 10 meat quality traits in intact and minced samples of loins from the same Iberian pig line, using the full near infrared region. NIRS technique could offer the opportunity to simultaneously quantify or categorise a large number of samples for the set of meat quality traits, with a single run, being a quick and clean technique. The results of these analyses were evaluated in terms of coefficient of determination (R^2), root-mean-square error (RMSE), residual prediction deviation (RPD) and range error ratio (RER). The highest coefficient of determination in external validation (R^2p) for intact loin samples were achieved for hardness, redness (a*) and yellowness (b*) ($0.7<R^2p<0.8$); while for minced samples lightness (L*), myoglobin content and texture parameters obtained always $R^2p>0.7$. The statistical models developed suggest the ability of NIRS for the quantitative prediction of shear force in minced samples. Moreover, a rough classification is possible of both cohesiveness in minced samples and colour parameters (L*, a*, b*) and hardness in intact samples.

The aim of the third and the fourth experiment was to analyse the differentially expressed genes between divergent groups for two meat quality traits through RNA-seq technique. Two groups of Iberian pigs with divergent breeding values for tenderness (experiment 3) and myoglobin content (experiment 4) were selected to characterize their longissimus dorsi transcriptomes. The regulations of biological processes, metabolic pathways and functions that involved differentially expressed genes and that can affect the variability of phenotypic expression in both traits were studied. Regarding the analyses of divergent groups for tenderness, the functional analyses showed an enrichment of differentially expressed genes in biological processes related to proteolysis (*CTSC*, *RHOD*, *MYH8*, *GADD45B*, *ACTC1*, *CASQ2*, *CHRNA9*), skeletal muscle tissue development (*ANKRD1*, *DMD*, *MSTN* and *FOS*), lipid metabolism (*FABP3* and *PPARGC1A*) and collagen metabolism (*COL14A1*). The differential expression analyses between divergent groups for myoglobin content revealed an enrichment of differentially expressed genes in biological process related to oxygen transport (*HBA1*), lipid metabolism (*ECH1*, *PLA2G10* and *PLD2*), inflammation (*CHST1*, *CD209* and *PLA2G10*) and immune system (*CD209*, *MX2*, *LGALS3*, *LGALS9, SLA1* and *SLA7*).

The results obtained in this thesis revealed that the genotyping data of *PRKAG3_rs319678464G > C*, *PRKAG3_rs330427832C > T* SNPs and *CAPN1_rs81358667G > A* will probably be able to be used to select sires and sows for water losses (*PRKAG3*) and tenderness (*CAPN1*). In addition, transcriptome analyses not only revealed two sets of candidate gene, one for tenderness and one for myoglobin content, but also increased the insight about the biological processes and networks underlying both traits. Lastly, the NIRS approach was shown as a promising tool for classifying intact meat samples into three categories according to their hardness and as a potential substitute for the Warner-Bratzler technique. In general, the results of this thesis will be very useful in Iberian pig production and open new tracks for the study and application of molecular information from omics technologies in this breed.

INTRODUCCIÓN

1. Introducción

1.1. El cerdo Ibérico: Origen y características

El cerdo (*Sus scrofa domestica*) es un mamífero de la familia *Suidae* que evolucionó de una progresiva y gradual domesticación del jabalí salvaje. Se originó en Asia y Mesopotamia hace alrededor de 10.000 años, aunque posteriormente hubo domesticaciones paralelas de jabalíes en otras zonas de Europa y del mundo (Larson et al., 2005; Groenen et al., 2012) que produjeron modificaciones fenotípicas en el color, tamaño y comportamiento de los animales (Diamond, 2002; Fang et al., 2009).

A partir de su domesticación, los diversos sistemas de producción y la adaptación al entorno dieron lugar a las razas porcinas locales, entre ellas la Ibérica. Ya en la Europa de los siglos XVII y XVIII, el aumento de la demanda del consumo de carne produjo un cambio en la producción con el desarrollo de estrategias de cría modernas, surgiendo nuevas razas porcinas con una mejor aptitud cárnica debido a un mayor desarrollo muscular y menor acumulación grasa. A finales del siglo XVIII se generaron cruces de cerdos del norte de Europa con razas procedentes de Asia, combinando el gran tamaño de los primeros con la mayor precocidad inicial y contenido graso de los segundos (White et al., 2011).

En la conocida descripción del cerdo Ibérico y de su sistema de producción, López-Bote (1998) incluyó al cerdo Ibérico entre las razas porcinas autóctonas europeas caracterizadas por no haber sufrido una selección productiva intensiva. Además, a diferencia de otras razas porcinas europeas, muestra una cierta singularidad genética puesto que diversos estudios realizados con marcadores moleculares han mostrado la ausencia de introgresión procedente de razas asiáticas o de otras razas comerciales europeas. (Alves et al., 2003; SanCristobal et al., 2006; Ollivier, 2009). Hasta los primeros años de este siglo, el cerdo Ibérico se consideraba una agrupación racial (Laguna-Sanz, 1998; Diéguez, 2000) debido a su gran heterogeneidad morfológica que procedía de una estructura tradicional de tipo reticulada (Dobao et al., 1987), con variedades locales distribuidas por todo el territorio en pequeños núcleos de población o ganaderías y con escaso intercambio de reproductores entre ellas. Muchas de estas variedades locales, diversas sobre todo en cuanto al color de la capa y cantidad de pelo, desaparecieron o

vieron muy mermados sus efectivos en la crisis poblacional entre las décadas de 1960 y 1980 que se extendió hasta la década siguiente, causada por la peste porcina africana, por los cambios de hábitos alimenticios y por la utilización masiva e incontrolada de cruces con Duroc. Sólo algunas de estas variedades permanecen en nuestros días. Esta circunstancia se refleja en las cinco variedades o estirpes reconocidas oficialmente en el Libro Genealógico de la raza (Figura 1): Torbiscal, Lampiño y Manchado de Jabugo, como variedades de protección especial, y Retinto y Entrepelado como variedades de fomento (Real Decreto 45/2019). De entre ellas, cabe destacar por su origen la estirpe Torbiscal, puesto que proviene de un cruce dialélico realizado en la piara experimental de "El Dehesón del Encinar" (Oropesa, Toledo), a partir de cuatro líneas ancestrales de los años 40: las coloradas portuguesas Ervideira (Évora) y Caldeira (Elvas), y las negras lampiñas extremeñas Campanario y Puebla (Odriozola, 1976), esta última aún mantenida en pureza con un elevado nivel de consanguinidad bajo el nombre de Guadyerbas (Toro et al., 2000). Por su parte, el Manchado de Jabugo procede de cruces realizados hace muchos años entre cerdos Ibéricos de la provincia de Huelva y algunas razas inglesas (Alves et al., 2008). Esta presencia de acervo genético procedente de razas inglesas ha sido un sólido argumento utilizado por varios grupos de investigación oponiéndose a incluir el Manchado de Jabugo en el Libro Genealógico como variedad de Ibérico, aunque finalmente el Ministerio responsable autorizase esta inclusión. La variedad Retinta es mayoritaria hoy día, aunque junto con el Entrepelado (originalmente producto del cruce de variedades negras con retintas) configuran una población mucho más homogénea que en el siglo pasado debido a la masificación de la producción y al cambio de la estructura reticular por otra de tipo más piramidal, con diversas ganaderías de élite que suministran reproductores al resto. La presente tesis doctoral se ha realizado con datos procedentes de animales de una ganadería tradicional de variedad Retinta.

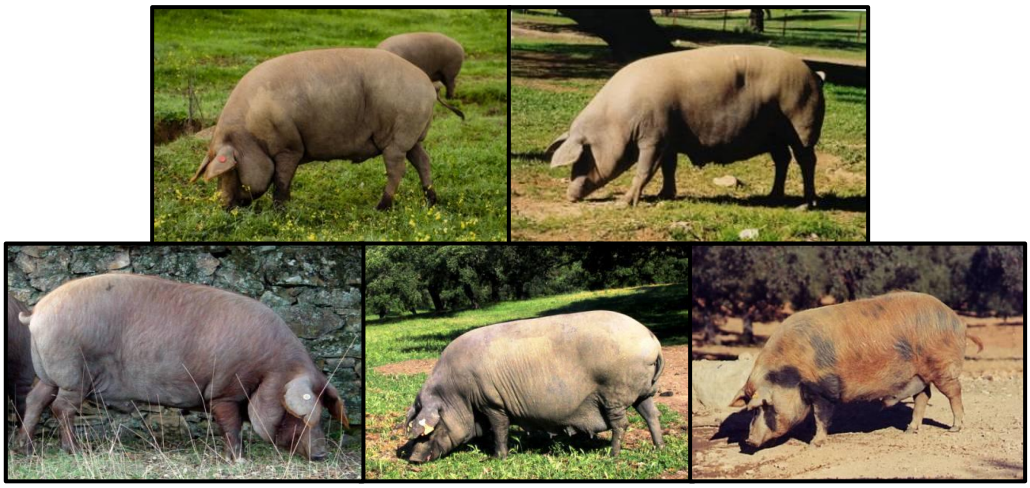


Figura 1. Estirpes de cerdo Ibérico reconocidas oficialmente (Retinto, Entrepelado, Torbiscal, Lampiño y Manchado de Jabugo. Fuente: mapa.gob.es).

La población de cerdo Ibérico se concentra fundamentalmente en el suroeste de la península Ibérica, donde está completamente integrado desde hace siglos en un ecosistema, la dehesa, que es resultado de la transformación del bosque mediterráneo por la acción ganadera del hombre. Este proceso de adaptación implica su rusticidad y resiliencia al clima y a la geografía (López-Bote, 1998). En la dehesa aprovecha perfectamente los recursos naturales, principalmente bellotas, pastos y raíces. En este sentido, cuando la carga ganadera se ajusta al territorio disponible y el manejo ganadero es el adecuado (Rodríguez-Estévez et al., 2012), el sistema productivo extensivo de la montanera se caracteriza por ser sostenible, en el que los animales disponen de mucho terreno, aprovechan estos recursos naturales y realizan un moderado y continuo ejercicio al tener que desplazarse para beber y alimentarse. Por ello se puede afirmar que, en el caso del cerdo Ibérico, este aprovechamiento de los recursos de la dehesa durante la etapa final del cebo constituye un magnífico ejemplo de integración entre un animal y su ecosistema. Este agroecosistema está formado por árboles del género *Quercus*, como encinas, robles y alcornoques que proporcionan bellotas, además de pastos que complementan una dieta excesivamente energética. La dehesa, que se puede definir también como un sistema mediterráneo silvopastoril tradicional que liga la producción y la conservación de la naturaleza (Olea et al., 2006), se extiende por 5,6 millones de hectáreas en el suroeste de la península Ibérica, repartidas entre 4 millones de hectáreas en España y 1,6 millones en el Alentejo portugués, y supone el principal recurso medioambiental y paisajístico de muchas de estas regiones de la península. La

producción de cerdo Ibérico y de otras razas vacunas y ovinas, es primordial para su conservación.

Además de por su rusticidad y por su adaptación al medio, al igual que otras razas porcinas autóctonas y en contraposición con las razas magras más seleccionadas, el cerdo Ibérico se caracteriza fenotípicamente por un alto potencial adipogénico, resultado de su alta capacidad de ingesta voluntaria (López-Bote, 1998). Esto ha sido relacionado con la expresión del gen *LEPR* (Óvilo et al., 2010). También posee una alta capacidad de infiltración muscular y de desaturación de grasa, lo que unido a su dieta de bellotas durante el cebo se traduce en un mayor contenido en ácidos grasos monoinsaturados, especialmente en ácido oleico. El porcino Ibérico se caracteriza por su menor prolificidad respecto a otras razas (López-Bote, 1998), además de por una alta tasa de síntesis y de degradación proteica (Rivera-Ferre et al., 2006), posiblemente relacionada con un menor desarrollo muscular (Óvilo et al., 2014).

Los cerdos ibéricos, en el manejo tradicional de montanera, nacen durante el verano y las primeras semanas del otoño, con prolongadas etapas de crecimiento, denominadas recría o pre-montanera, en las que sufren una alimentación sumamente restringida desde el punto de vista de la cantidad diaria suministrada. Este manejo tiene como objetivo conseguir que los animales lleguen a la montanera (fase de cebo) con un buen desarrollo anatómico, pero sin excesiva grasa corporal, especialmente sin acumulación de grasa subcutánea. Así, el cerdo llega hasta los 90-110 kg de peso con una edad de 11 a 15 meses, pudiendo alcanzar ese peso en manejos muy tradicionales a los 16-18 meses. En los últimos 3-4 meses del periodo de montanera, el animal repone entre 40 y 60 kg de peso, casi todo en forma de tejidos grasos. Finalmente, el peso de sacrificio se realiza en torno a los 150-160 kg, con una edad de 14 a 18 meses o incluso mayor.

El objetivo de mejorar la prolificidad, la tasa de crecimiento e incrementar el contenido magro de las canales, condujo al ensayo de cruces con diversas razas foráneas en la segunda mitad de siglo XX (Dobao et al., 1986). De estos ensayos, finalmente el cruce con Duroc se mostró como el más adecuado, posiblemente por su mayor proximidad racial con el ibérico debido a su origen (Jones, 1998), manteniéndose este

cruce en la actualidad de forma masiva y siendo uno de los mayores responsables de la posterior intensificación de la producción.

Todo ello ha dado lugar a la situación actual en la que la población de cerdos Ibéricos, criados en pureza o en cruces con Duroc y la utilización de diferentes sistemas productivos, conviven bajo la regulación de la Norma de Calidad de productos del cerdo Ibérico elaborados en España (Real Decreto 4/2014), modificado posteriormente para poder incorporar la utilización de animales de la raza Alentejana de Portugal, con el mismo origen, morfología y etología que la Ibérica y que recientes estudios genéticos han demostrado la enorme similitud entre ambas (Muñoz et al., 2018a). La norma de Calidad, que obliga a utilizar siempre madres Ibéricas, tiene el objetivo de definir, identificar y certificar la calidad de dichos productos, empleando una serie de definiciones y requisitos según el tipo racial del animal de sacrificio (100%, 75% o 50% Ibérico) y el sistema de producción (de bellota o montanera, de cebo de campo y de cebo intensivo). El etiquetado final de los productos resume las distintas categorías en cuatro: *bellota 100% Ibérico* (precinto negro), *bellota Ibérico* (cruzado al 75% o al 50%, precinto rojo), *cebo de campo Ibérico* (precinto verde) y *cebo Ibérico* (precinto blanco), estos dos últimos pueden ser indistintamente 100% Ibéricos o cruces al 75% o 50% (Figura 2).

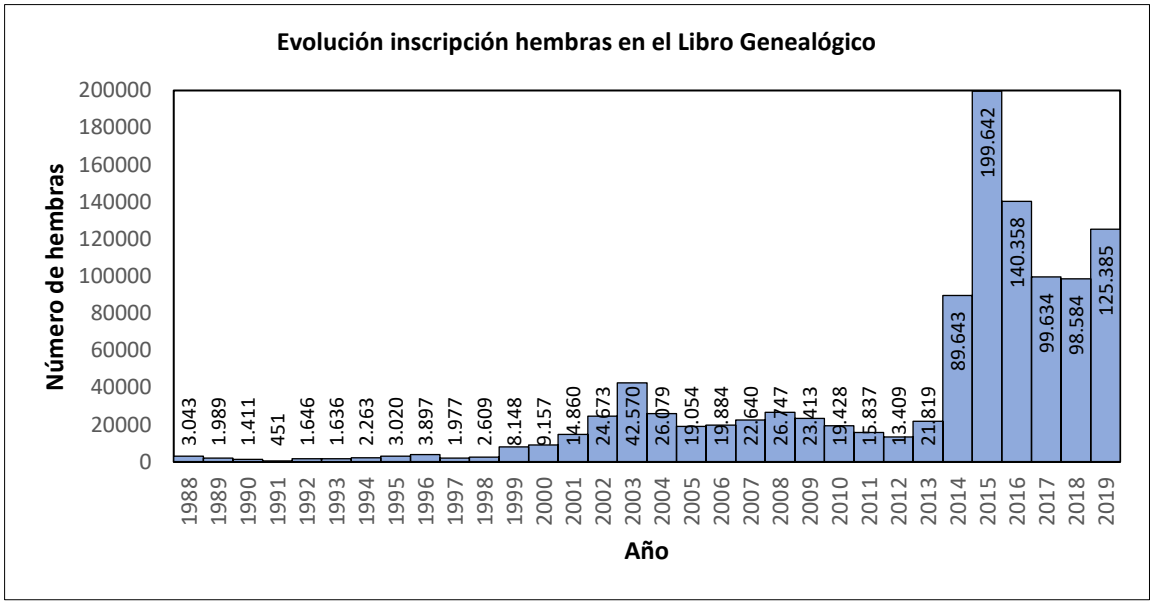
Figura 2. Precintos para identificación de categorías de productos Ibéricos, norma calidad 4/2014 (Fuente: ASICI).

1.2. Datos productivos y censo

El sector porcino español ocupa el cuarto lugar a nivel mundial, sólo detrás de China, EEUU y casi al mismo nivel que Alemania (Fuente: EUROSTAT, USDA y Comisión Europea. Elaboración: SG Producciones Ganaderas y Cinegéticas. www.mapa.gob.es). En España supone casi un 42% del total de la producción ganadera y algo más del 16% de la producción agraria, con datos del año 2019 (Fuente: SG Análisis, coordinación y Estadística. www.mapa.gob.es). El grado de crecimiento en la producción cárnica de cerdo ha sido tal, que la tasa de autoabastecimiento ha ido aumentando progresivamente hasta llegar al 188,1% (año 2019), por lo que la exportación de carne porcina se ha convertido en un motor fundamental de la economía, con un 53% de exportaciones respecto al total de la producción (año 2019), siendo China el principal socio de mercado (Fuente: DataComex (AEAT) www.mapa.gob.es).

El cerdo Ibérico representa el 10,8% del censo porcino nacional (año 2019. Fuente: S.G. Análisis, Coordinación y Estadística (MAPA), Elaboración: SG Producciones Ganaderas y Cinegéticas. www.mapa.gob.es), población que ha aumentado alrededor de un 2% desde la instauración de la norma de calidad (RD 4/2014). Este censo de porcino Ibérico históricamente ha sufrido fuertes oscilaciones (Espárrago et al., 1999), relacionadas con causas muy diversas (deforestación y cambios en la explotación agrícola-ganadera, modas de alimentación o graves enfermedades). Así, en la fuerte crisis de las décadas 1960-1980, la población de porcino Ibérico pasó por momentos críticos, en los que estuvo en serio riesgo de extinción. Sin embargo, en los últimos 20 años la producción de cerdo Ibérico ha experimentado un crecimiento casi continuo, si exceptuamos los años de la crisis económica de la pasada década y cierto estancamiento en este último año de pandemia. En diciembre de 2019, el censo inscrito en el Libro Genealógico era de 592.000 reproductores, de los cuales 584.000 eran hembras y 8.100, verracos (AECERIBER, 2020). Como la Norma de Calidad obliga a la inscripción de los reproductores en el Libro, este censo de algo más de 0,55 millones de cerdas puede considerarse indicativo del total de la raza. Las Figuras 3a y 3b muestran la evolución en los últimos años de las inscripciones de reproductores, donde destaca el considerable aumento durante los años 2015 y 2016 motivado, precisamente, por la citada reglamentación publicada en 2014 (RD 4/2014). Sin embargo, sí es importante destacar

los números muy bajos de cerdas inscritas en los años anteriores a 1999 y la evolución al alza a partir de entonces.



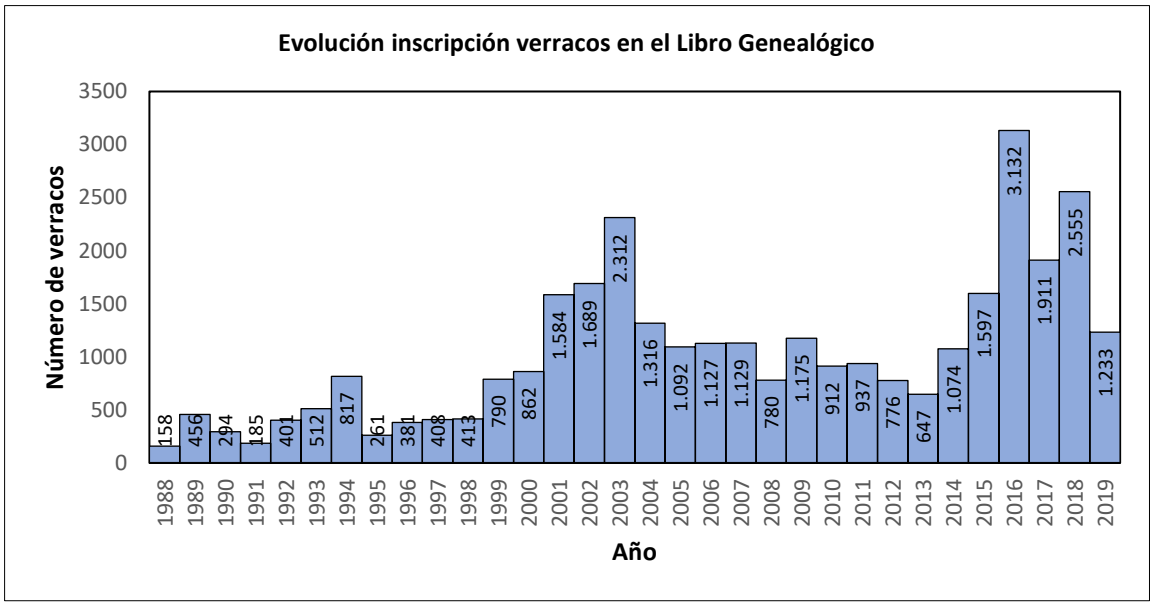


Figura 3a) y 3b). Evolución de las inscripciones de machos y hembras en el libro genealógico (1988 – 2019). Fuente: AECERIBER.

Los datos de cerdos Ibéricos totales sacrificados desde 2013 se representan en la Figura 4, donde se indica además el número de los que fueron clasificados como 100% bellota (precinto negro), y describen una situación de continuo crecimiento, con casi 1,5 millones de sacrificios más en estos 8 años. Además, el efecto de la Norma de Calidad 2014 respecto al etiquetado exclusivo de los cerdos Ibéricos puros de bellota se refleja en la importancia creciente de su porcentaje respecto al total: de 5,7% en 2013 se incrementó a 9,5% en 2020. En este año 2021, los datos de la montanera indican que,

en la categoría de los cerdos de bellota, los 100% ibéricos han aumentado respecto a años anteriores hasta suponer el 63% de la producción total de este tipo de alimentación. El 37% restante se reparte entre 75% y 50% ibérico, con el 9% y el 28%, respectivamente (Fuente: ASICI).

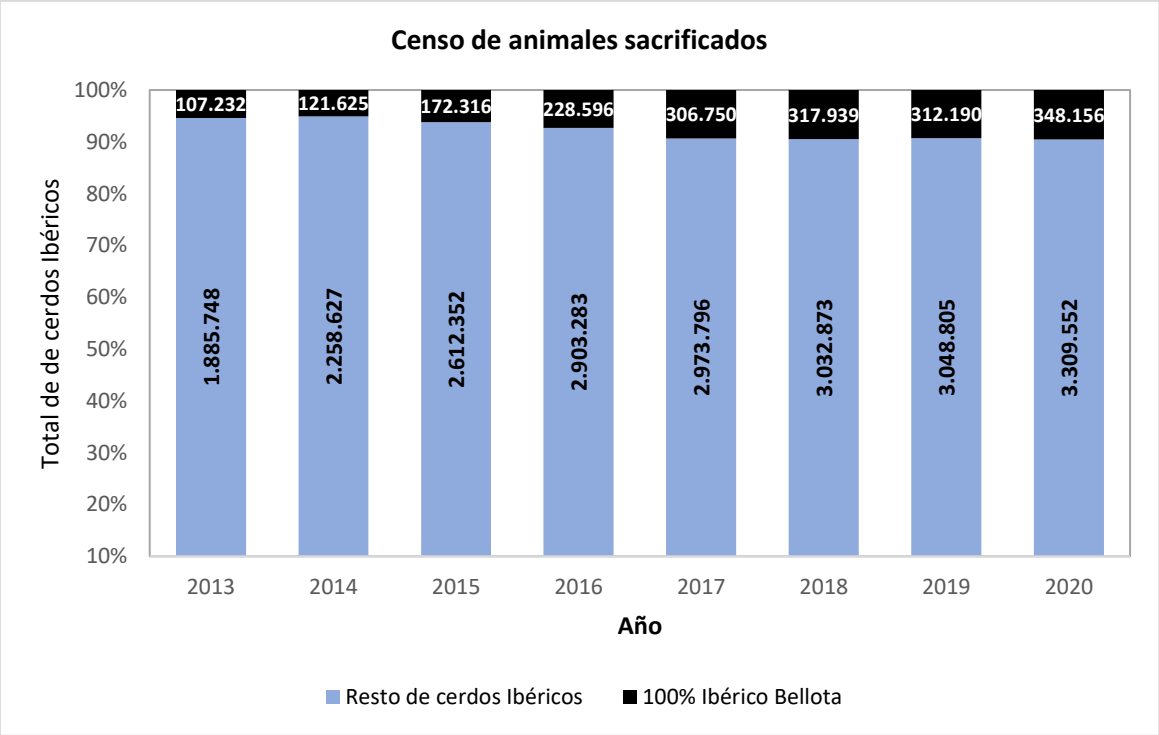


Figura 4. Datos productivos del censo de animales sacrificados (2013-2020. Fuente: Registro General Informativo de Organismos Independientes de Control del Ibérico, RIBER).

Una visión global de los distintos tipos de producción (sistemas de alimentación y porcentaje de raza ibérica que conviven bajo la normativa de calidad, RD 4/2014), en términos numéricos, se presenta en la Tabla 1, con el número de cerdos sacrificados y su porcentaje en cada categoría en el año 2019. Estos datos describen una situación en la que apenas el 15% fueron cerdos Ibéricos criados en pureza y de éstos, el 9,3% correspondió a cerdos alimentados en montanera (de bellota, precinto negro). Es decir, la mayor parte de la producción, casi el 85%, fueron cerdos cruzados, bien al 50% bien al 75%. Es importante señalar que el porcentaje de cerdos cruzados de bellota, aunque ligeramente, también supera al de puros, (9,52% vs 9,29%). Cerca del 63% del total de los animales se criaron bajo el sistema de cebo en intensivo (precinto blanco) y de ellos el 58% eran cruzados al 50%, la categoría, por raza y alimentación, claramente mayoritaria. La Tabla 1 pone de relieve que el cebo de campo va alcanzando mayor relevancia, estando ya en el 18%, y que el escaso número de cerdos cruzados al 75% (no

llega al 5%) pone en cuestión el mantenimiento en la Norma de esta categoría racial que requiere de un complejo control documental de los verracos cruzados del 50% y que, además, produce cerdos genética y morfológicamente muy heterogéneos.

Tabla 1. Número de cerdos Ibéricos sacrificados y su porcentaje en función del sistema de alimentación y del tipo racial (diciembre 2019, Fuente: Registro General Informativo de Organismos Independientes de Control del Ibérico, RIBER).

% Ibérico	Bellota	Cebo de campo	Cebo	Total
100%	9,29%	2,06%	3,76%	15,11%
75%	2,46%	1,50%	0,62%	4,58%
50%	7,06%	15,05%	58,20%	80,31%
Total	18,81%	18,61%	62,58%	**3.360.995 (100%)**

Finalmente, los datos de productos certificados comercializados siguiendo la clasificación por precintos de colores correspondiente al RD 4/2014 se presenta en la Figura 5. Además de los ya citados 100% Ibéricos de bellota (precinto negro) y de los alimentados en cebo intensivo, sea cual sea su porcentaje racial (precinto blanco), las estadísticas incluyen el total de productos de cerdos cruzados de bellota (precinto rojo) y de cebo de campo (precinto verde).

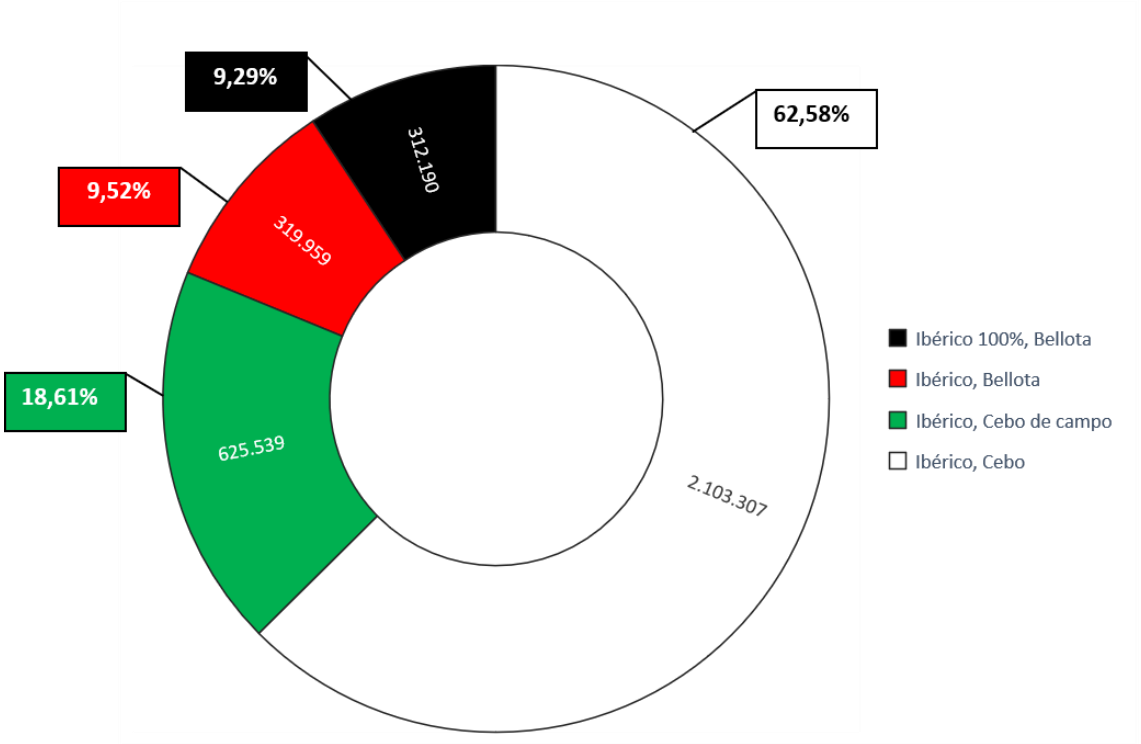


Figura 5. Datos totales de comercialización de productos cárnicos Ibéricos, según precintos RD 4/2014. (diciembre 2019, Fuente: Registro General Informativo de Organismos Independientes de Control del Ibérico, RIBER).

1.3. Calidad de los productos

En el sector agroalimentario, la calidad se define como el conjunto de propiedades y características de un producto relativas a las materias primas utilizadas en su elaboración, a su naturaleza, composición, pureza, identificación, origen y trazabilidad. Además, engloba a los procesos de elaboración, almacenamiento, envasado, comercialización y etiquetado (Ley 28/2015). En este sentido, surge la necesidad de medir, cuantitativa o cualitativamente, la calidad desde un punto de vista tecnológico y objetivo.

Como calidad de la carne se pueden considerar cuatro acepciones: calidad tecnológica, calidad organoléptica, calidad nutricional y calidad higiénico-sanitaria.

1.3.1. Calidad tecnológica

La calidad tecnológica es muy relevante para la industria cárnica puesto que está asociada con un adecuado procesado de la materia prima. Entre los parámetros relacionados con esta calidad, cuatro de los más importantes son el pH, la capacidad de retención de agua, la estabilidad oxidativa (Maltin et al., 2003) y el contenido en grasa intramuscular. Respecto al pH, la medida de la bajada post-mortem en la carne, es un aspecto crucial, por lo que se realiza a los 45 minutos y a las 24 horas después del sacrificio. Valores de pH anómalos pueden dar lugar a carnes con atributos de calidad no deseables, las conocidas como pálidas, blandas y exudativas (PSE) u oscuras, duras y secas (DFD) (Listrat et al., 2006; Adzitey & Nurul, 2011). El segundo parámetro, la capacidad de retención de agua de la carne, es también relevante porque se ha descrito que carnes con menores pérdidas de agua mostraron una mayor terneza (Melody et al., 2004; Lonergan & Lonergan, 2005). Este carácter puede ser medido mediante las pérdidas de agua por descongelado, cocinado, goteo y fuerza centrífuga.

La estabilidad oxidativa es otro de los parámetros importantes en los procesos de la industria cárnica. Desde el punto de vista tecnológico, se ve afectada por la composición del perfil lipídico. Los ácidos grasos poliinsaturados son más propensos a la oxidación, por lo que un mayor contenido en la carne puede tener un efecto no deseable en su estabilidad oxidativa, afectando al color, causando rancidez que incide negativamente en el flavor, ocasionando mayores pérdidas de agua y, en definitiva, un

acortamiento de la vida útil del producto cárnico (Wood et al., 2008). Estos mismos autores describieron que la vitamina E en la suplementación del animal, tiene un efecto protector sobre la oxidación de la carne, fundamental para mejorar la estabilidad oxidativa de los ácidos grasos poliinsaturados.

Finalmente, el contenido en grasa intramuscular tiene un enorme protagonismo en los largos procesos tecnológicos de los productos cárnicos curados (Ventanas et al., 2006), como jamones, paletas o lomos. Así mismo, la grasa intramuscular juega un papel fundamental en la consistencia, firmeza y cohesividad de la carne para su consumo en fresco. Además, es el principal carácter de calidad desde el punto de vista organoléptico.

1.3.2. Calidad organoléptica

Las características organolépticas de la carne son aquellas que se perciben a través de los sentidos, como son la apariencia, la textura y el flavor. El primer estimulo que percibimos de un producto cárnico es su apariencia: tamaño, forma o color.

Dentro de estos parámetros, cabe destacar el contenido en grasa intramuscular, el cual está relacionado con la mayor parte del resto de caracteres de calidad. Así, se ha observado una relación entre el mayor contenido en grasa intramuscular con menores valores de dureza y fibrosidad, ambos atributos de textura (Ruiz & López-Bote, 2002). También está relacionado con la terneza, la jugosidad (Tejeda et al., 2002) y con la retención de agua. De hecho, la jugosidad podría estar más asociada al nivel de grasa intramuscular que al propio contenido en agua (López-Bote, 2001). Es, además, el principal responsable del sabor de los productos frescos e influye de manera importante en el sabor final de los productos curados.

El contenido en grasa intramuscular se ve afectado por factores como el tipo de músculo, la genética (tanto la raza como el propio individuo), el sistema de producción (alimentación), la edad y el sexo. La extracción y medida del porcentaje graso en carne se puede realizar siguiendo el método clásico de Bligh & Dyer, 1959 o, más recientemente, mediante tecnología NIRS (Solís et al., 2001; Zamora-Rojas et al., 2011). En este sentido, la metodología NIRS puede ser de gran utilidad para remplazar algunos métodos tradicionales de análisis físico-químicos, ya que no requiere destruir la muestra y se evita el uso de reactivos químicos (Solís et al., 2001; Cáceres-Nevado et al., 2019). Esta tecnología sería válida bien para cuantificar el carácter de forma precisa (Ripoll et

al., 2008), bien para clasificar las muestras en categorías en función del contenido (Monroy et al., 2010). Por otro lado, puesto que no es sencillo medir ciertos caracteres de calidad en carne mediante NIRS debido a la composición heterogénea de músculo y a sus propiedades físico-químicas peculiares (Cozzolino et al., 2000), esta metodología necesita de una adecuada y laboriosa calibración para asociar la absorción espectral de la región del infrarrojo cercano con los registros obtenidos en el laboratorio.

En los diversos trabajos publicados sobre el contenido de grasa intramuscular en lomo fresco de cerdo Ibérico, se observa una elevada variabilidad, oscilando las medias en un rango de entre el 3% y 9,8% (Rey et al., 2001; Fernández et al., 2003; Alves et al., 2005; Óvilo et al., 2006; Daza et al., 2008; García-Casco et al., 2008; Tejerina et al., 2012a; Ayuso et al., 2013; García-Casco et al., 2014; Ibáñez-Escriche et al., 2016). Posiblemente este amplio rango esté motivado por las diferencias existentes entre tratamientos e individuos, pero también por el lugar de toma de la muestra en el propio lomo (apical, central o caudal). En la presente tesis doctoral, la media del contenido en grasa intramuscular medida mediante NIRS en muestras tomadas en el centro del lomo en los 1.199 cerdos Ibéricos de montanera fue de 4,9 %, con un rango de 1,7 % a 9,8 %.

Respecto al resto de caracteres citados que influyen en la calidad organoléptica, el color, se considera una de las más importantes, influyendo en las preferencias de decisión de los consumidores (Mancini y Hunt, 2005). De este modo, las carnes que no son pálidas, sino que muestran un color cereza rojo brillante son elegidas por los consumidores entre otras razones porque son indicativas de salubridad (Yu et al., 2017). Los caracteres de calidad relacionados con el color pueden ser medidos objetivamente mediante colorímetro (valor L* para luminosidad, valor b* amarillez y valor a* para rojizo). El color rojo de la carne está asociado con el contenido en el pigmento mioglobina y los posibles estados químicos en los que se encuentre: deoximioglobina, oximioglobina, metamioglobina y carboximioglobina (Mancini & Hunt, 2005). El contendido en mioglobina de la carne es, por lo tanto, también un carácter de calidad relevante (Ventanas et al., 2005), el cual se puede medir de manera objetiva mediante el método de Hornsey (1956).

La textura es otro de los caracteres más importantes de calidad de la carne y engloba parámetros como la terneza, jugosidad, *masticabilidad* (propiedad mecánica relacionada con el tiempo o número de masticaciones necesarias para su deglución) o

cohesividad (propiedad mecánica relativa al grado de deformación antes de su rotura). Durante el proceso de transformación del músculo en carne, ocurren una serie de mecanismos físico-químicos. Entre ellos, la proteólisis juega un papel fundamental en la terneza final de la carne (Kemp et al., 2010), ya que está relacionada con el grado de alteración de las proteínas y la estructura del músculo post-mortem (Hopkins & Taylor, 2004). Proteasas como calpaínas, calpastatinas, catepsinas, caspasas y quinasas están implicadas en este proceso de maduración de la carne (Kemp et al., 2010) durante el cual las catepsinas degradan la fuerte interacción de los enlaces de actina-miosina, debilitándolos y produciendo el ensanchamiento de los sarcómeros (unidad funcional contráctil de las miofibrillas musculares). En este punto, las calpaínas tienen mayor capacidad de hidrolizar las proteínas asociadas, lo que influye en la maduración del músculo (Kemp et al., 2010; Li et al., 2012). La textura puede ser medida de forma objetiva mediante texturómetro por varios métodos: la prueba de resistencia al corte mediante sonda Warner-Bratzler, la prueba de compresión-relajación (SR test) o la prueba de análisis del perfil de textura (TPA). En la textura de la carne pueden influir también parámetros como el contenido en grasa intramuscular, el perfil de ácidos grasos, el contenido en colágeno y la capacidad de retención de agua.

1.3.3. Calidad nutricional e higiénico-sanitaria

La calidad nutricional se centra en el estudio de los nutrientes que puede aportar la carne, como los macronutrientes (proteínas, hidratos de carbono y lípidos), y los micronutrientes (vitaminas y minerales). Finalmente, la calidad higiénico-sanitaria es la que se refiere a la inocuidad del producto, libre de contaminación biótica o abiótica y sin riesgo sanitario. Los métodos para asegurar esta calidad se basan en una correcta trazabilidad y un sistema de análisis de peligros y puntos de control crítico (APPCC).

La idoneidad de la materia prima entre estas cuatro acepciones no siempre es unidireccional y puede llevar a contradicciones entre algunas de ellas. Por ejemplo, para la calidad organoléptica y tecnológica un cierto contenido en grasa intramuscular es positivo, además un adecuado contenido en sal es necesario desde el punto de vista de la calidad tecnológica en los procesos de curación. Sin embargo, desde un enfoque nutricional, tanto el contenido en grasa como el de sal pueden resultar negativos para la salud, como se ha evidenciado en la reciente propuesta de clasificación de los

productos curados del cerdo para la etiqueta Nutri-score (Szabo et al., 2019) que ha generado cierta polémica y confusión en el sector agroalimentario en general y del cerdo Ibérico en particular.

1.3.4. Calidad y cerdo Ibérico

Como se ha indicado previamente, la raza Ibérica se caracteriza por tener un mayor grado de engrasamiento de la carne contribuyendo de manera positiva a su calidad organoléptica (López-bote, 1998). Por ello, la carne y los productos cárnicos procedentes de la raza Ibérica están estrechamente relacionados con la calidad, asociándose con un color rojo intenso característico, con un mayor grado de infiltración grasa y con un perfil lipídico donde predominan los ácidos grasos monoinsaturados. En este sentido, diversos estudios han descrito que carnes procedentes de cerdos Ibéricos mostraron mayor valor del parámetro a* (color rojo) y menor valor L*(palidez) que la carne de Ibéricos cruzados o de otras razas porcinas convencionales (Serra et al., 1998; Estévez et al., 2003; Serrano et al., 2008). Diversos trabajos también describieron mayores valores de contenido en grasa intramuscular medida en músculo longissimus de cerdo Ibérico (Serra et al., 1998; Estévez et al., 2003; Serrano et al., 2008) o mayor nivel de veteado en jamones y lomos curados (Carrapiso et al., 2003; Ventanas et al., 2007). Finalmente, la grasa de cerdo Ibérico se caracteriza por un mayor contenido en ácidos grasos monoinsaturados, principalmente oleico (López-Bote, 1998; Tejerina et al., 2012a) y en antioxidantes (Rey et al., 2006), relacionado esto último con el sistema extensivo de producción.

Este último aspecto resulta crucial para los caracteres de calidad de la carne, ya que la producción de cerdo en extensivo destaca por el lento crecimiento de los animales y, generalmente, por el mayor peso final de engorde en comparación con los sistemas intensivos. Por lo tanto, el elevado peso y edad contribuyen a favorecer determinados atributos de calidad, como por ejemplo el color o la deposición de grasa intramuscular (Bonneau & Lebret, 2010). Además, se ha descrito que el tono rojo cereza apreciado por los consumidores en la carne está asociado a un mayor contenido en mioglobina y a músculos con mayor proporción de fibras musculares oxidativas, característico de cerdos criados en sistemas extensivos, debido al mayor ejercicio que realizan (Ventanas et al., 2005).

En definitiva, la apreciada calidad organoléptica de la carne de cerdo Ibérico criado en extensivo (montanera), están determinada por el equilibrio perfecto que forman la combinación de sus características genéticas y el sistema extensivo de producción tradicional.

1.4. Programas de mejora genética en cerdo

Los principales conocimientos científicos que sustentan la mejora genética animal se remontan a finales del siglo XIX y principios del XX, pero el inicio de su aplicación masiva en la producción animal puede fijarse en los años posteriores a la Segunda Guerra Mundial. A partir de ese momento, hubo un punto de inflexión en los sistemas agroalimentarios occidentales causado por la necesidad de producir alimentos (proteína animal, en este caso) de manera rápida y eficaz. Esto dio lugar a una estrategia de producción a gran escala basada en la intensificación y la especialización. El sector porcino fue uno de los mayores protagonistas en el intento de superar la emergencia alimentaria europea, viéndose reflejado en programas de mejora enfocados a la producción de cerdos de mayor velocidad de crecimiento y con mayor aptitud cárnica, lo que derivó en un abandono de otras muchas razas locales consideradas menos rentables (Čandek-Potokar et al., 2019). El éxito de las razas mejoradas con respecto al crecimiento cárnico influyó de manera negativa en la calidad organoléptica de la carne, al ser los caracteres de tipo productivos (incremento en magro, por ejemplo) antagónicos con algunos caracteres de calidad (porcentaje de grasa intramuscular), algo que ya avanzaron estudios como el de Barton-Gade (1990) sobre la experiencia en razas magras seleccionadas (Large White, Landrace, Duroc y Hampshire), y Silió et al. (1992) acerca de *lo que es posible y lo que es deseable* en la selección de cerdos Ibéricos.

Sin embargo, a partir de la década de los 70, el desarrollo económico alcanzado en el continente, la satisfacción de la demanda de carne y las nuevas inquietudes ambientales y animalistas, han propiciado el resurgir del interés por la conservación de las razas porcinas autóctonas, asociadas a un sistema de producción más benévolo con el animal y sostenible con el entorno, y a unos atributos de calidad de sus productos cárnicos tradicionales. Estos aspectos inherentes a estas razas son muy apreciados actualmente por los consumidores más exigentes. Por ello, el éxito de las razas locales y sus sistemas de producción se basan en este trinomio formado por su peculiaridad

genética, su adaptación a un entorno geográfico y productivo concretos, más ajustada a las especificaciones de bienestar animal y, finalmente, a la calidad diferenciadora de sus productos. En este contexto, el cerdo Ibérico y por extensión el Alentejano, constituyen el principal ejemplo a nivel europeo del éxito económico de una raza y de sus productos (Čandek-Potokar et al., 2019).

Los programas de selección genética buscan la mejora de caracteres de interés, que se denominan objetivo de selección, y que son distintos en función de la especie e incluso de las razas dentro de una misma especie. Para realizar este proceso se seleccionan como reproductores los mejores individuos para el carácter que se desea mejorar desde el punto de vista genético, y no solo fenotípico. De esta forma, estos individuos dejan un mayor número de descendientes a la siguiente generación, modificando el carácter en el sentido deseado y produciendo lo que se conoce como respuesta a la selección.

En los programas de mejora convencionales, para poder elegir a estos reproductores se miden los caracteres directamente en los propios individuos y/o en individuos emparentados, aprovechándose en la evaluación genética de toda la información familiar disponible de la población donde se implementa el programa de mejora. Con esta información, utilizando modelos de análisis y métodos estadísticos adecuados, se estiman los valores aditivos o mejorantes. Desde hace ya varias décadas el método conocido como BLUP (del inglés Best Lineal Unbiased Prediction), es el comúnmente más aceptado para la estima de estos valores mejorantes (Henderson, 1984). Las estimas BLUP, obtenidas mediante el ajuste de las observaciones aplicando el denominado Modelo Animal que incluye la matriz de todas las relaciones de parentesco, están corregidas o ajustadas por los efectos de valor fijo de tipo intrínseco (como por ejemplo el sexo) y de manejo o ambiental (como pueden ser el rebaño o la campaña), utilizando toda la información genealógica disponible (Kennedy, 1990). La metodología BLUP-Modelo Animal permite, por tanto, estimar el valor mejorante de todos los individuos de la matriz de parentesco, aunque no se disponga de datos fenotípicos (Henderson 1984).

Estos métodos se siguen utilizando de manera rutinaria en la mayoría de los programas de mejora, han sido muy útiles y han conseguido mejorar considerablemente

una serie de caracteres reproductivos, de crecimiento y de composición de la canal cuando han sido aplicados en las razas más comerciales, de tal forma que a esas razas se las denomina ahora como *cerdos magros*.

Sin embargo, estos métodos tienen una serie de limitaciones especialmente relativas a la adquisición de los datos fenotípicos, ya que algunos de los caracteres sólo pueden ser medidos *postmortem* en animales emparentados, generalmente coetáneos o descendientes. Además, debido a que hacen falta una gran cantidad de registros, en el caso de caracteres en los que para su medición se utilizan determinaciones laboriosas y costosas, la implementación de programas de mejora tradicionales pueden resultar económicamente discutibles. Finalmente, en especies de parto múltiple, como el cerdo, los hermanos de la misma camada sin registros que sean candidatos a la selección tienen los mismos valores mejorantes, aunque no son genéticamente idénticos, por lo que se pierde eficacia a la hora de seleccionar.

La aparición de las técnicas de genética molecular durante las últimas décadas del siglo XX ha permitido desarrollar una serie de herramientas genéticas y genómicas que han sido propuestas, en un principio, para solventar estas limitaciones de los programas de mejora clásicos. A continuación, se explica su evolución y aplicación en mejora genética en porcino.

1.4.1. Uso de información molecular en programas de mejora

En un primer momento, el uso de la genética molecular en programas de mejora genética se basó en dos aproximaciones que están íntimamente relacionadas: a) el gen candidato y b) el mapeo genético de loci de caracteres cuantitativos (QTL, Quantitative Trait Loci). La primera se basa en la idea de que un gen que codifica para una proteína implicada funcionalmente en la variación del carácter, puede albergar una mutación que altere la expresión fenotípica del mismo. Los primeros experimentos que indagaron en esta aproximación secuenciaban los genes candidatos, buscaban polimorfismos y posteriormente se calculaba si existía una asociación estadística con los caracteres. Un ejemplo de esta aproximación es la identificación de una sustitución en el gen *PRKAG3* asociada al contenido en glucógeno del músculo, que se relacionaba con carne con un bajo pH final y con menor capacidad de retención de agua (Milan et al., 2000).

La diferencia con la estrategia de mapeo de QTL es que se identifican regiones del genoma cuya variación alélica está asociada con la variación de un carácter cuantitativo asumiendo que los genes que afectan al carácter son desconocidos. Para llevar a cabo esta técnica en un principio se elegían marcadores neutrales de tipo microsatélite y se elaboraban mapas genéticos para posteriormente reconocer los QTL (Andersson et al., 2001). Uno de los primeros estudios de detección de QTL se realizó en un cruce de jabalí y Large White sobre parámetros de crecimiento y deposición grasa (Andersson et al., 1994).

La estrategia de mapeo de QTL y gen candidato no son excluyentes si no que se pueden combinar, llevando a cabo la detección de QTL en un primer paso para posteriormente buscar genes candidatos funcionales que estén localizados dentro de la región del QTL, aumentando así la robustez del gen como candidato. Un ejemplo claro de esta combinación de aproximaciones es el caso de la detección de la mutación causal detectada en el gen *IGF2* que tiene efecto sobre el crecimiento magro (Van Laere et al., 2003). No obstante, los QTL detectados utilizando marcadores de tipo microsatélites tienen unos amplios intervalos de confianza (Nagamine et al., 2003), lo que puede dificultar la identificación de genes candidatos y las potenciales mutaciones causales de la variación fenotípica. Para poder disminuir estos intervalos de confianza se propuso disponer de una mayor densidad de marcadores y mapas en los que se pueda delimitar con una mayor precisión su posición (Fan et al., 2010).

La aplicación final de los estudios de gen candidato y QTL consiste en utilizar esta información molecular para poder seleccionar los animales genéticamente superiores para un carácter a través de su genotipo. A esta aproximación se la conoce como selección asistida por marcadores (MAS, Marker-assisted selection), de la se pueden establecer tres tipos:

1) Gene-MAS o GAS: Selección de la mutación causada por el efecto del QTL.
2) LE-MAS: Basada en el equilibrio de ligamiento del marcador con el QTL.
3) LD-MAS: Basada en el desequilibrio de ligamiento entre QTL y marcador.

En la Tabla 2 se presenta un resumen del número de QTL identificados en la actualidad asociados a caracteres de calidad de carne relevantes.

Tabla 2. Número de QTL identificados hasta el momento (junio 2021) en el genoma porcino en función de los caracteres de calidad (Fuente PigQTLdb).

Tipo de carácter	Número de QTL identificados
Capacidad de retención de agua	34
Pérdidas de agua por cocinado	91
Pérdidas de agua por descongelado	20
Pérdidas de agua por goteo	1092
Color L* (CIELAB)	117
Color a*(CIELAB)	200
Color b*(CIELAB)	122
Pigmentos (hematina)	12
Contenido en mioglobina en músculo	2
Resistencia al corte (texturómetro)	205
Puntuación en terneza	76
pH (45 min post mortem)	116
pH (24 h post mortem)	260
Nivel de veteado	127
Contenido en grasa intramuscular	786
Ratio ácidos grasos monoinsaturados/saturados	62
Contenido en ácido oleico	792
Contenido en ácido linoleico	193

Este tipo de selección solventaría los problemas mencionados anteriormente de la selección clásica o tradicional en el caso de los caracteres de calidad, en los que son necesarias costosas mediciones fenotípicas *post-mortem* en animales emparentados. Sin embargo, no ha podido ser extensamente aplicada debido a sus inconvenientes: grandes intervalos de confianza de los QTL y a que, en la mayoría de los casos, la parte de la varianza del carácter que es explicada por los marcadores moleculares es escasa.

A principios de la década de los 2000 se constituyó el consorcio para la secuenciación del genoma porcino (SGSC, Swine Genome Sequencing Consortium) que supuso un hito en el desarrollo de la genómica porcina (Groenen, 2016). La primera versión del genoma porcino se llevó a cabo utilizando cromosomas artificiales de bacterias (BACs) (Archibald et al., 2010). Desde entonces, se ha revisado y mejorado, apareciendo sucesivas versiones incorporando información de secuenciación de genoma completo, culminando en la actual Sscrofa 11.1 que data del año 2017 (Li et al.,

2017) y que incluso ha sido recientemente revisada utilizando información de secuenciación masiva utilizando fragmentos largos (Warr et al., 2020).

Las técnicas de secuenciación masiva han permitido no sólo mejorar la secuenciación del genoma porcino, sino que también ha propiciado la aparición y desarrollo de nuevas técnicas genómicas que se están aplicando en la mejora genética. Una de estas técnicas se basa en el genotipado masivo de polimorfismos de un solo nucleótido (SNPs) utilizando chips que contienen un número elevadísimo de éstos. En el mercado hay disponibles hasta el momento tres tipos de chips de genotipado para cerdos: SNP60 v2 Genotyping BeadChip de Illumina con 64.232 SNPs, GGP-Porcine HD de Neogen con 70.231 SNPs y Axiom Porcine Genotyping Array de Affymetrix con 658.692 SNPs. Las principales aplicaciones de este genotipado masivo son los análisis de asociación de genoma completo (GWAS, Genome-wide associations studies) y la selección genómica.

Los estudios de tipo GWAS permiten asociar estadísticamente los registros fenotípicos con la información obtenida a partir del genotipado utilizando los chips de genotipado masivo (Goddard & Hayes, 2009). Como resultado, se detectan regiones QTL con intervalos de confianza inferiores a los observados con datos obtenidos a partir de microsatélites, lo que ha facilitado la búsqueda de genes y mutaciones causales en porcino que pueden ser usadas como criterio de selección en programas de mejora. El uso de GWAS ha permitido identificar miles de nuevas asociaciones entre regiones genómicas y caracteres de calidad relevantes (Ponsuksili et al., 2014; Liu et al., 2015; Zhang et al., 2015) en cruces porcinos como son Pietrain × (Landrace × Large White), Erhualian y Duroc × (Landrace × Yorkshire), y Duroc × (Landrace × Large White), respectivamente.

La otra gran técnica genómica por excelencia es la selección genómica de extensa aplicación en algunas especies ganaderas. El fundamento de esta aproximación consiste en la estimación de los valores aditivos o mejorantes utilizando la información de una cantidad masiva de SNPs a lo largo de todo el genoma (Meuwissen et al, 2001). La selección genómica aprovecha el desequilibrio de ligamiento y asume que, si utilizamos la información de un gran número de marcadores genéticos, alguno debe estar en desequilibrio de ligamiento con el QTL. Para poder aplicar la selección genómica el

procedimiento que se sigue consiste en utilizar los datos de genotipos y fenotipos de una población grande de referencia para estimar los efectos de los genotipos en el carácter. Posteriormente estos efectos se utilizan en una ecuación predictiva en la cual se pueden estimar los valores mejorantes de los individuos sin necesidad de conocer sus fenotipos. La selección genómica ha sido muy exitosa en la raza de vacuno de leche Holstein; sin embargo, es de difícil aplicación en otras especies y razas debido a la ausencia de una buena población de referencia y de conexión entre ganaderías, además del posible menor grado de desequilibrio de ligamiento en esa raza o población determinada. Por ello, las particularidades genéticas de cada raza deben ser tenidas en cuenta para para valorar la consecución del éxito en su aplicación (Ibáñez-Escriche & González-Recio, 2011). Finalmente, la inversión económica que requiere la selección genómica debe también ser tenida muy en cuenta, puesto que, en poblaciones pequeñas, poco estructuradas o con potencial económico limitado su implementación puede ser desaconsejable.

Otra fuente de información relevante que puede ser utilizada en programas de mejora genética proviene del conjunto genes expresados en un tejido concreto y en un momento puntual de la edad o estado del individuo, que se conoce como transcriptoma. Los estudios más comunes se basan en analizar las diferencias de expresión entre grupos divergentes de individuos. En un primer momento, se desarrollaron los microarrays de expresión que son bases sólidas con miles de sondas fijadas que permiten analizar la expresión de un número determinado de genes conocidos. El desarrollo de la tecnología de microarrays fue una herramienta con la que se pudo avanzar en el progreso e implementación de técnicas de análisis para caracterizar transcriptoma de diferentes tejidos (Pena et al., 2014). No obstante, en los últimos años, los métodos denominados de nueva generación han permitido la secuenciación del transcriptoma mediante la técnica conocida como RNA-seq. Utilizando esta técnica se puede estudiar el transcriptoma de un tejido mediante un análisis más exhaustivo, ya que, a diferencia de los microarrays en los que se analizaba sólo una parte de los tránscritos capturados, con RNA-seq podemos analizar todos los tránscritos expresados en el tejido y además obtener otros resultados, como la detección de nuevas isoformas no descritas hasta la fecha (Chen et al., 2011). Además, desde el punto de vista técnico, el RNA-seq es una

técnica más sensible que los microarrays de expresión requiriendo menor cantidad de ARN de partida, no tiene tantos problemas de ruido como los microarrays ya que estos últimos pueden presentar problemas de hibridación cruzada y, además, presenta una menor variación técnica (Oshlack et al., 2010).

El procedimiento general del RNA-seq (Figura 6) consiste en la fragmentación del ARN y posterior secuenciación de fragmentos cortos de entre 35 y 500 pb mediante las tecnologías disponibles en el mercado, siendo la de Illumina la más utilizada (Martin & Wang 2011). Una vez secuenciados los fragmentos, se pueden alinear y ensamblar usando un genoma de referencia o ensamblarse *de novo*. Después del ensamblaje de estos tránscritos, su expresión puede ser cuantificada gracias a que el número de lecturas de cada tránscrito es proporcional a su nivel de expresión. Una vez se determina este perfil de expresión se puede llevar a cabo el análisis estadístico de sus diferencias cuantitativas (Oshlack et al., 2010). Posteriormente, se llevan a cabo análisis funcionales *in-silico* en los que se emplean una serie de herramientas bioinformáticas para analizar en qué procesos y funciones biológicas, rutas metabólicas y redes génicas de interacción están implicados los genes diferencialmente expresados. Por último, en la mayoría de los estudios publicados se sigue realizando la validación técnica de los resultados de RNA-seq mediante PCR cuantitativa (qPCR). No obstante, esta validación sigue siendo a día de hoy objeto de debate en la comunidad científica en cuanto a la necesidad de hacerla o no, ya que la propia técnica de RNA-seq se muestra más robusta que la qPCR (Coenye, 2021).

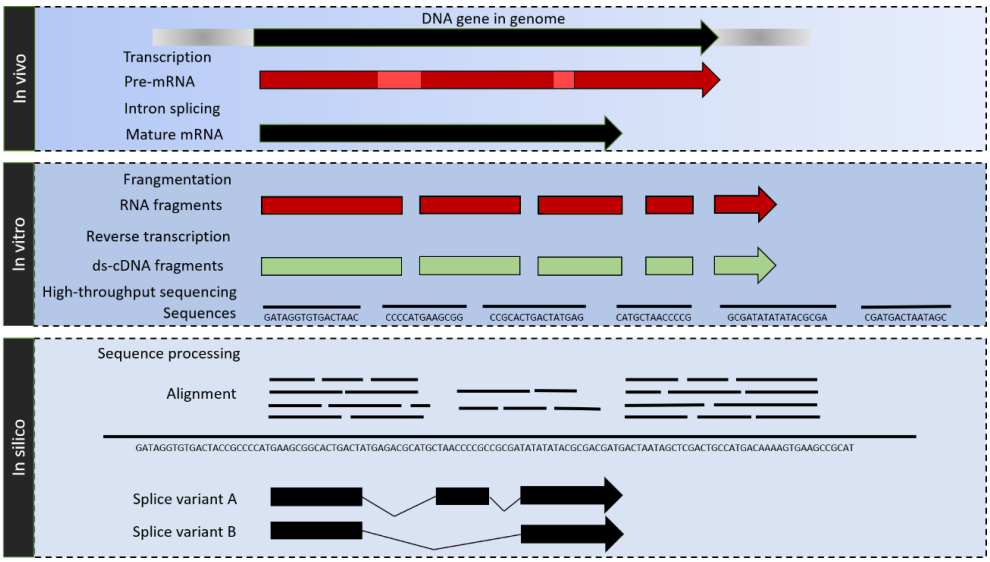


Figura 6. Diagrama de representación de un experimento RNA-seq.

En la última década se han publicado numerosos estudios centrados en el análisis del transcriptoma para cerdos con características divergentes en cuanto a un determinado carácter o fenotípico, o pertenecientes a dos razas *a priori* con características diferentes entre sí (Tabla 3). Así, por ejemplo, se ha comparado el análisis de expresión diferencial de razas autóctonas y de razas de alto crecimiento magro (Damon et al., 2012; Hamill et al., 2012; Horodyska et al., 2018; Zappaterra et al., 2020).

Tabla 3. Listado de estudios en porcino realizados con la técnica RNA-seq y relacionados con caracteres de calidad.

Referencia	Raza	Tipo de tejido
Albuquerque et al., 2021	Alentejano y Bísaro	Músculo longissimus lumborum
Núñez et al., 2021	Mangalitsa y Moravka	Músculo longissimus dorsi
Villaplana-Velasco et al., 2021	Ibérico	Músculo longissimus thoracis
Albuquerque et al., 2020	Alentejano y Bísaro	Grasa subcutánea dorsal
Zappaterra et al., 2020	Large White	Músculo semimembranosus
Benítez et al., 2019	Ibérico y Duroc	Grasa subcutánea
Horodyska et al., 2018	Landrace x Large White	Músculo longuissimus thoracis
Muñoz et al., 2018b	Ibérico	Músculo longissimus dorsi
Piórkowska et al., 2018	Pulawska y Landrace	Musculo longissimus y semimembranosus
Xu et al., 2018	Wey y Yorkshire	Músculo longissimus
Cardoso et al., 2017	Duroc	Músculo gluteus medius
Wang et al., 2017	Rongchang	Músculo longissimus y grasa subcutánea
Li et al., 2016a	Wannanhua y Yorkshire	Músculo longissimus dorsi
Li et al., 2016b	Pietrain x Duroc x Landrace x Yorkshire	Músculo longissimus dorsi
Zhu et al., 2016	Large White	Músculo soleus
Corominas et al., 2015	Ibérico x Landrace	Hígado, grasa dorsal y músculo longissimus
Puig-Oliveras et al., 2014	Ibérico x Landrace	Músculo longissimus dorsi

1.4.2. La mejora en el Cerdo Ibérico

La mayoría de los programas de selección porcina se han enfocado hacia la mejora de parámetros productivos y reproductivos, como son el índice de crecimiento, la ganancia media diaria, la composición magra, el número de lechones nacidos vivos o la tasa de partos. En este sentido, el éxito de la selección efectuada durante años en muchas razas está perfectamente probado (Hill, 2008). Sin embargo, las experiencias de mejora genética en cerdo Ibérico comenzaron muy tarde y hasta la fecha no hay demasiados estudios en los que se diseñe y analice la implementación de estos programas, y mucho menos para caracteres de calidad de carne. Todo ello puede estar relacionado con la estructura tradicional de la población y con la especificidad del sistema de producción en extensivo.

Como se ha descrito anteriormente, la mayoría de las razas porcinas autóctonas europeas se caracterizan por haber sido escasamente sometidas a la presión de procesos selectivos sistemáticos en caracteres productivos. Por supuesto, esta característica se cumple fielmente en el caso del cerdo Ibérico, ya que salvo una selección ganadera empírica orientada a potenciar determinadas características morfológicas, relacionadas con un patrón racial, o las fisiológicas concernientes a la rusticidad o la capacidad de engrasamiento, los programas de mejora han estado por completo ausentes hasta los años 90 del siglo pasado. Desde el punto de vista de la evaluación genética y la selección, las primeras iniciativas se llevaron a cabo por el grupo de porcino del Departamento de Mejora Genética de INIA, cuyas experiencias se trasladaron de manera casi simultánea al Programa Oficial de Mejora de la Raza, impulsado desde el Ministerio de Agricultura, Pesca y Alimentación de la época con la publicación de los reglamentos del esquema de valoración genética para la raza porcina Ibérica (1992 y 2011), auspiciados, ejecutados y gestionados por la Asociación Española de Criadores de Cerdo Ibérico (AECERIBER).

1.4.2.1. La experiencia de INIA en *"El Dehesón del Encinar"*

La piara experimental de *El Dehesón del Encinar* (Odriozola, 1976; Silió & Rodrigáñez, 2013) fue creada en los años 40 del siglo pasado en las dehesas de la localidad de Oropesa (Toledo), como ya se ha indicado, con ejemplares de cuatro

estirpes ancestrales. La localización de esta finca ofrecía situación geográfica idónea para la época, por su cercanía a Madrid teniendo en cuenta las malas comunicaciones en esos años, por el aislamiento sanitario obligado por la peste porcina africana y por las características climáticas y ambientales de la dehesa del suroeste peninsular. A partir de la creación en los años 60 de la estirpe genética Torbiscal, se contó con un material biológico muy adecuado para afrontar diversos estudios de conservación y selección genéticas, llevados a cabo por el Dpto. de Mejora Genética de INIA (Béjar et al., 1992). Entre ellos, a finales de los años 80 se inició un proyecto sobre *Aplicación de nuevas metodologías a la conservación y selección de poblaciones porcinas españolas* que incluyó una experiencia de evaluación y selección mediante metodología BLUP. En ella, a partir de dos líneas de Torbiscal, *control* y *selección*, se comprobó la respuesta a la selección para la información fenotípica registrada (pesos y espesor de tocino dorsal medios a los cuatro meses de edad) cuando se elegían mediante sus valores mejorantes a los reproductores de la línea de selección (Toro et al., 1995). En esta experiencia se aplicó por primera vez en cerdo Ibérico las evaluaciones genéticas mediante BLUP-modelo animal, obteniéndose además las primeras estimas de parámetros genéticos y ambientales para los caracteres implicados mediante máxima verosimilitud restringida (REML) (Meyer, 1991). La presión selectiva ejercida a partir de los valores mejorantes estimados, mostró una respuesta moderada con diferencias significativas entre ambas líneas para crecimiento, proporción de hueso y magro, espesor de grasa dorsal y porcentaje de grasa intramuscular en lomo, a favor de la línea de selección en los dos primeros caracteres, y a favor de la línea control en los dos segundos (Silió et al., 1997; Silió & Rodrigáñez, 2013). Es decir, se comprobó por primera vez en esta raza el antagonismo existente entre caracteres productivos (crecimiento y rendimiento en magro) y de calidad (grasa intramuscular).

1.4.2.2. El esquema de valoración genética oficial de la raza

A partir de las experiencias de *El Dehesón del Encinar*, entrados los años 90, mediante una colaboración INIA-AECERIBER se iniciaron las experiencias de evaluación genética del programa oficial de la raza. El programa de mejora actual puede visitarse en http://www.aeceriber.es/documentos.html. Este fue un momento clave en relación con la selección del Ibérico, puesto que fue, y sigue siendo, el principal intento de

involucrar a la población en su conjunto en actividades de mejora genética. El diseño inicial de este programa buscaba un doble objetivo. Por un lado, conseguir una mejora de los caracteres de crecimiento registrando pesos a edades tempranas (Rodríguez et al., 2000), obligando así al ganadero de extensivo a un control genealógico que era muy inusual en esa época. Por otro, aprovechar estos ejemplares con genealogía para desarrollar pruebas de ciclo completo realizadas en una misma finca de montanera (AECERIBER, 1998). De esta manera se efectuaban valoraciones BLUP intra-ganadería del peso a 90 días y valoraciones conjuntas del crecimiento en montanera y rendimiento en piezas nobles. Estos segundos análisis permitían la comparación entre ganaderías a través de los efectos fijos del modelo animal, puesto que la ausencia de suficientes conexiones genealógicas impedía comparaciones genéticas entre reproductores de distintas explotaciones. Posteriormente, se incluyó el porcentaje de grasa intramuscular e incluso el perfil de ácidos grasos como caracteres de calidad (Fernández et al., 2003), puesto que se era consciente del riesgo que podría suponer la selección basada exclusivamente en las piezas nobles (Silió et al., 1992; Silió, 2000).

Con estos primeros pasos del programa de mejora se intentaba convencer al ganadero tradicional de Ibérico de la necesidad de un eficiente control de rendimientos mediante registros y de un riguroso control genealógico, como requisitos indispensables para llevar a cabo una adecuada selección y obtener una mejora genética. A partir de estos trabajos y los posteriores, se efectúan las evaluaciones genéticas que desembocan en los Catálogos de Sementales que publica AECERIBER anualmente (http://www.aeceriber.es/noticias/catalogo-de-sementales-de-la-raza-porcina-iberica-2020.html). Además, los diversos resultados se han ido publicando en la revista Sólo Cerdo Ibérico (Silió et al., 1999; Rodríguez et al., 2000; Ureta et al., 2010) o en congresos y revistas científicas (Fernández et al., 2003; García-Casco et al., 2014), poniendo de manifiesto las moderadas estimas de heredabilidad para los caracteres de peso de lechón a edades tempranas y las elevadas estimas para los caracteres de composición de la canal, que asegurarían respuestas positivas mediante la selección basada en el programa. En estos primeros años, los caracteres reproductivos (tamaños de camada, aptitud materna) fueron excluidos en el programa puesto que su baja heredabilidad, el tamaño pequeño de las explotaciones y la falta de conexiones genealógicas sumado a la

dificultad de homogeneizar estos registros entre ellas, aconsejaron concentrar los esfuerzos sólo en los demás objetivos (Silió, 2000). Actualmente, el programa oficial sí incluye estos caracteres, aunque algunos de los problemas indicados aún persisten.

1.4.2.3. La Genética molecular en los programas de mejora del cerdo Ibérico

Como se ha mencionado antes, la selección genómica ha sido muy exitosa especialmente en la raza Holstein de vacuno. En porcino, aunque se han predicho aumentos de beneficio desde el 10% (Lillehammer et al. 2013) hasta el 50% (Knol et al. 2016) en caracteres maternos, su aplicación práctica en cerdos Ibéricos resulta desaconsejable. La ausencia de una población de referencia de gran tamaño, las aún escasas conexiones genealógicas entre ganaderías y el elevado coste de genotipado de los chips de alta densidad, son argumentos que hacen inviable que esta estrategia se pueda aplicar de manera práctica en la mejora de esta raza de población limitada. Por ello, en un programa de mejora de la raza Ibérica podría plantearse la utilización de marcadores genéticos asociados a caracteres de relevancia, como herramienta de apoyo a la selección. Sin embargo, la aplicación de marcadores genéticos debe hacerse con cautela debido a la singularidad genética de la raza, al sistema productivo y también al moderado antagonismo genético descrito entre caracteres productivos y de calidad, como es el caso del rendimiento en piezas nobles y el contenido en grasa intramuscular (Fernández et al., 2003), ya que una selección intensiva en función de los primeros seguramente llevaría a valores no deseables para los segundos, en detrimento de la calidad de carne. Desde este punto de vista, se han propuesto esquemas de selección centrados en la mejora de parámetros productivos como el rendimiento de piezas nobles (jamón, paleta y lomo) buscando a su vez no perjudicar las características de calidad cárnicas (Silió, 2000; García-Casco et al., 2014).

Los primeros trabajos de genética molecular en los que estaban implicados individuos de la raza Ibérica comenzaron en la década de los 90 con la formación de los consorcios IBMAP y MEIBMAP en los que estaban implicados investigadores del INIA, IRTA y UAB. Estos trabajos estaban orientados a la detección de regiones cromosómicas relacionadas con caracteres de crecimiento, composición corporal, calidad de carne (contenido en grasa) y maternos. Dentro del consorcio IBMAP se desarrolló en primer lugar el cruce experimental Ibérico x Landrace (F2, F3, RC1 y RC2) enfocado a los estudios

de crecimiento, composición corporal y calidad de carne utilizando principalmente datos de microsatélites y polimorfismos en genes candidatos (Óvilo et al., 2000; Óvilo et al., 2002; Clop et al., 2003; Muñoz et al., 2007). En esta etapa se realizaron grandes progresos con los estudios en genes como *LEPR* (Óvilo et al., 2005; Muñoz et al., 2009) y *ELOVL6* (Corominas et al., 2013). Dentro del MEIBMAP se realizó un nuevo cruce experimental entre hembras de la raza hiperprolífica Meishan y verracos Guadyerbas, orientado a la búsqueda de QTL para caracteres reproductivos y el análisis de los posibles genes candidatos subyacentes (Rodríguez et al., 2005; Noguera et al., 2009; Fernández-Rodríguez et al., 2010).

Posteriormente, en los proyectos y trabajos del consorcio IBMAP se generaron otros dos más a partir de dos cruces Ibérico x Duroc e Ibérico con Pietrain, y además se comenzaron a usar datos de genotipado masivo en estos dos retrocruces y en uno de Ibérico x Landrace. Esta estrategia permitió disminuir los intervalos de confianza de los QTL previamente encontrados, detectar nuevos y llevar a cabo análisis de asociación genómica para detectar regiones genómicas y SNPs concretos asociados fundamentalmente a caracteres de crecimiento (Fernández et al., 2012) y composición de ácidos grasos (Ramayo-Caldas et al., 2012; Muñoz et al., 2013; Martínez-Montes et al., 2018; Crespo-Piazuelo et al., 2020).

La información genómica recopilada permitió obtener medidas nuevas de diversidad genética y consanguinidad de las estirpes (Silió et al., 2013). Además, durante las dos primeras décadas del siglo XXI, se iniciaron y continuaron algunos estudios de secuenciación masiva del genoma porcino Ibérico (Esteve-Codina et al., 2011).

Por último, los trabajos más recientes se han enfocado hacia análisis del transcriptoma mediante RNA-seq y secuenciación. Algunos de estos estudios se realizaron en cerdos Ibéricos y su cruce con razas como Duroc o Landrace (Óvilo et al., 2014; Pérez-Montarelo et al., 2014; Puig-Oliveras et al., 2014; Ayuso et al, 2016; Benítez et al., 2019). Sin embargo, los trabajos con muestras procedentes de cerdos Ibéricos puros criados en sistemas en extensivo (montanera) todavía no son muy numerosos (Muñoz et al., 2018b), aunque la información genómica que aportan es muy apropiada para su utilización en programas de mejora.

Siguiendo la senda investigadora construida por los trabajos anteriores, algunas iniciativas más se han llevado o se están llevando a cabo por empresas como Sánchez Romero Carvajal-Jabugo (SRC) (Muñoz et al., 2018c) y la Fundación La Contienda (García-Casco et al., 2017, 2020), ambas en colaboración con INIA y basadas en información molecular, cuya finalidad es realizar evaluaciones genéticas para seleccionar cerdos con valores mejorantes para caracteres productivos y de calidad.

Además, cabe destacar los recientes trabajos del grupo Inga Food en colaboración con diferentes centros de investigación como el IRTA, la UAB y la UPV (Ibáñez-Escriche et al., 2016; Pena et al., 2019; Varona et al., 2020; de Hijas-Villalba et al., 2021; Villaplana-Velasco et al., 2021).

La presente tesis doctoral está orientada hacia la caracterización fenotípica y genética, así como hacia la búsqueda de genes candidatos y la aplicación de tecnologías -ómicas, en una línea de cerdo Ibérico cerrada (Muñoz et al., 2018c) para un conjunto de caracteres de calidad. El propósito final es considerar la posible inclusión de estos genes en un programa de mejora de cerdos Ibéricos de montanera. Los objetivos planteados en esta tesis y los distintos experimentos, se describen con detalle en los siguientes apartados.

OBJETIVOS

El objetivo general de la presente tesis doctoral consistió en la inclusión de la información molecular obtenida a partir de la aplicación de técnicas ómicas (genómica y transcriptómica) en un programa de mejora para cerdos Ibéricos de montanera centrado en caracteres de calidad y en el uso de nuevas técnicas de fenotipado. Para ello se caracterizó fenotípica y genéticamente una población de cerdos de una línea Ibérica cerrada desde hace años a la entrada de reproductores externos. Para el estudio se consideraron y se determinaron los siguientes caracteres de calidad de carne: pérdidas de agua por descongelado, cocinado y por fuerza centrífuga (atributos de capacidad de retención de agua); color Minolta instrumental de luminosidad (L*), rojo (a*) y amarillo (b*) y contenido en mioglobina (atributos de color); medida instrumental de textura de la resistencia al corte y fuerza de compresión (atributos de terneza).

Los objetivos específicos fueron:

1. Caracterización de la población para un conjunto de caracteres de calidad de carne a partir de muestras de músculo longissimus dorsi; estimación de los parámetros genéticos (heredabilidades y correlaciones genéticas); evaluación de los efectos potenciales de un panel de SNPs mapeados en genes candidatos, diseñado específicamente para caracteres de calidad esta raza, mediante un análisis de asociación entre genotipos y fenotipos **(Experimento 1).**
2. Evaluación de la precisión de la técnica NIRS, usando la región completa del infrarrojo cercano, para determinar el conjunto de caracteres de calidad con el fin de valorar si se puede reemplazar en un futuro los análisis físico-químicos habituales (métodos de referencia) empleados en un laboratorio de calidad. La técnica NIRS podría ofrecer la oportunidad de cuantificar y cualificar diversos caracteres de calidad de carne para un elevado número de muestras, de manera rápida, limpia y de una sola vez. Esto sería además relevante en un programa de mejora, dado el elevado número de registros fenotípicos que se necesitan **(Experimento 2).**
3. Análisis de expresión diferencial mediante la secuenciación del transcriptoma completo del músculo longissimus mediante la técnica de RNA-seq, identificación de estos genes diferencialmente expresados y comprensión de su implicación en la regulación de procesos biológicos, rutas metabólicas y funciones que puedan afectar a la variabilidad de la expresión fenotípica de estos caracteres. Este análisis de

expresión diferencial se ha realizado en cerdos divergentes para los caracteres de resistencia al corte (**Experimento 3)** y contenido en mioglobina (**Experimento 4**). El propósito final ha sido proponer, si es posible, un conjunto de genes candidatos para los programas de mejora que podrían contener polimorfismos con efectos asociados a estos dos caracteres de calidad.

MATERIAL Y MÉTODOS

3.1. Material

3.1.1. Animales

Para la presente tesis se utilizaron muestras del músculo longissimus dorsi de cerdos Ibéricos, todos ellos machos castrados, procedentes de una línea comercial cerrada genéticamente desde hace más de 15 años propiedad de la empresa Sánchez Romero Carvajal – Jabugo S.A. (SRC). En el año 2012 se inició un proyecto para un programa de mejora en colaboración con INIA, que significó a partir de entonces un control genealógico y un registro de datos fenotípicos exhaustivos. Estos animales nacían entre el verano y el otoño en la finca *Monte Castilla* (La Granada de Riotinto, Huelva) y se trasladaban en la primavera del año siguiente a la finca *Los Agustinos* (Cazalla de la Sierra, Sevilla), donde completaban su etapa de crecimiento (recría y premontanera) hasta aproximadamente los 100 kg. El manejo en *Monte Castilla* era de tipo semi-extensivo mientras que en la finca *Los Agustinos* era extensivo, en ambos casos bajo un régimen de restricción alimentaria. A finales de octubre comenzaba la montanera en las dehesas de Cazalla de la Sierra con alimentación *ad libitum* a base de bellotas y pastos. La extensión de la finca es de aproximadamente 468 hectáreas repartidas en varias parcelas cercadas en las que se realizó el manejo clásico en este sistema, mediante el cual los cerdos se agruparon de acuerdo con su peso en dos o tres lotes de engorde, disponiéndose una rotación de estos lotes por las parcelas en función de la disponibilidad de bellotas. Desde 2014 a 2018, por cada campaña se cebaron aproximadamente unos 250-300 cerdos, es decir en cinco campañas consecutivas se controlaron durante la montanera unos 1250-1500 cerdos. Finalmente, cuando los animales alcanzaban el peso de sacrificio, eran sacrificados en el matadero de SRC en Jabugo (Huelva), con una edad aproximada de 17 meses y un peso de 165 kg. Durante estas cinco campañas se tomaron registros de crecimiento en campo y de despiece en matadero de 1.199 cerdos con genealogía completa, así como muestras de sangre y de distintos tejidos. La Figura 7 muestra esquemáticamente las campañas y el número de cerdos implicados en cada uno de los experimentos. En la figura 8 se representa una imagen de los cerdos tomada durante la montanera.

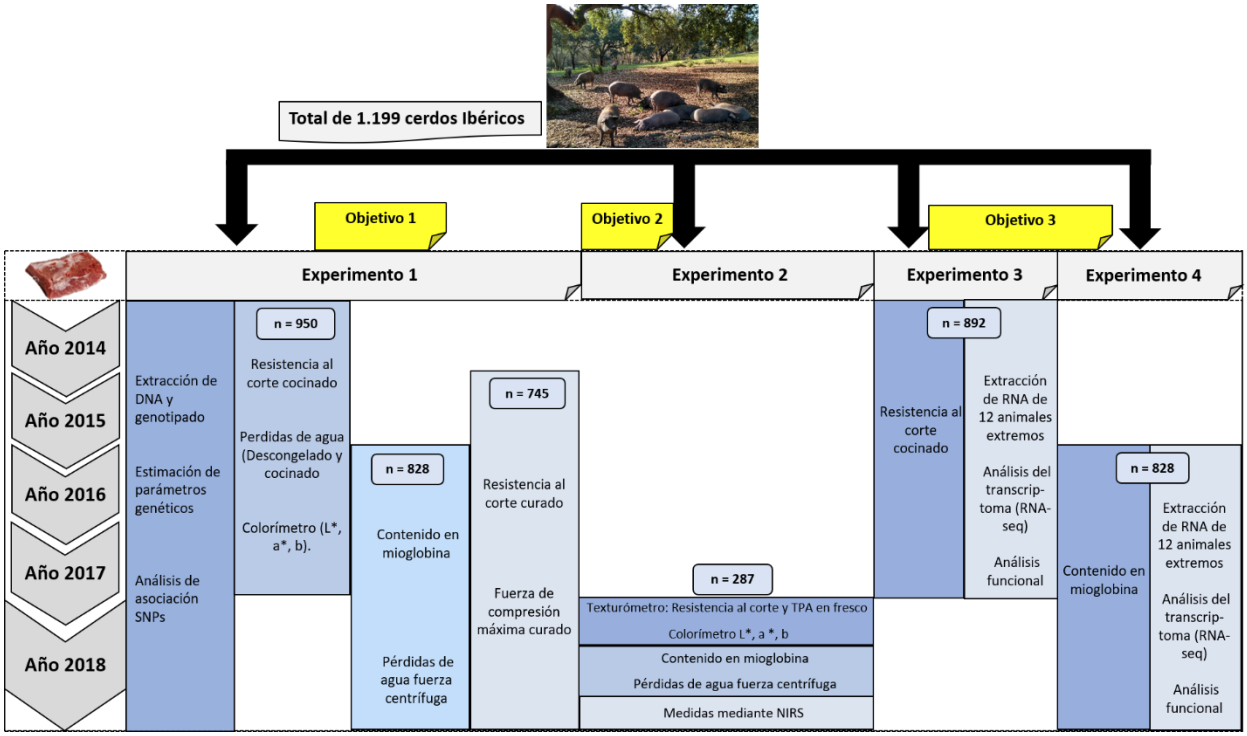


Figura 7. Diagrama de flujo del diseño experimental.

Todo ello se llevó a cabo bajo correctas prácticas siguiendo las normativas de manejo y protección animal: directiva 2010/63/EU y Real Decreto 53/2013 sobre la protección de animales usados en investigación. Además, los protocolos fueron evaluados y aprobados por el comité de ética en investigación animal del INIA.

Figura 8. Cerdos Ibéricos en montanera, en la finca *Los Agustinos* (Cazalla de la Sierra, Sevilla).

3.1.2. Muestras

De cada cerdo se tomó una porción de muestra de aproximadamente 700 gramos, procedentes del músculo longissimus dorsi (lomo). Como se representa en la Figura 9, estas muestras de lomo fresco fueron divididas en diferentes partes y envasadas a vacío, de acuerdo con los análisis y determinaciones de calidad que se llevarían a cabo posteriormente. Dependiendo del tipo de determinación posterior, algunas de estas submuestras fueron conservadas a -20ºC (determinaciones de pérdidas de agua por descongelado y cocinado, color y textura instrumental), o fueron introducidas en nitrógeno durante aproximadamente 20 segundos (contenido en mioglobina y pérdidas de agua por fuerza centrífuga). Además, se tomó un pequeño trozo del lomo de cada individuo que se introdujo en tubos criogénicos y se almacenó a -80ºC para la extracción del ARN y posterior análisis del transcriptoma.

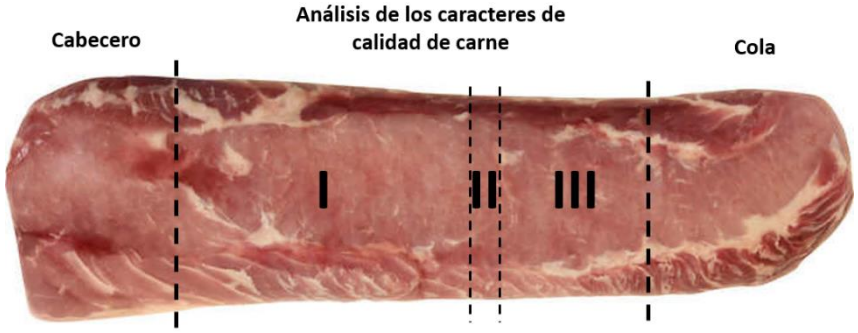


Figura 9. Determinaciones de calidad. Sección del músculo longissimus dorsi.

I: Pérdidas de agua por descongelado y cocinado, texturómetro (resistencia al corte muestras cocinadas).
Toma de muestra para RNA-seq.
Texturómetro en fresco (resistencia al corte y TPA).
Toma de muestra para NIRS.
II: Colorímetro (L*, a*, b*).
III: Contenido en mioglobina y pérdidas de agua por fuerza centrífuga.

El otro lomo de cada cerdo fue curado siguiendo el sistema tradicional de curación de productos del cerdo Ibérico, controlando su trazabilidad. Posteriormente en estas muestras se llevaron a cabo determinaciones de textura instrumental en producto curado.

3.2. Métodos

Los métodos de análisis se pueden dividir en cuatro tipos (Figura 7):

- Determinaciones de caracteres de calidad en laboratorio (Experimentos I, II, III, IV).
- Extracciones de DNA y su genotipado para un panel de 32 SNPs (Experimento I).
- Extracciones de RNA y obtención del transcriptoma mediante RNA-seq (Experimento III y IV).
- Análisis estadísticos y bioinformáticos:
  - Estimación de componentes de varianza y estimas de valores mejorantes (Experimento 1).
  - Análisis de asociación entre fenotipos y genotipos (Experimento 1)
  - Pretratamiento de los datos, calibración de NIRS y análisis quimiométrico (Experimento 2).
  - Análisis de expresión diferencial en muestras extremas y análisis funcional de los genes diferencialmente expresados (Experimento 3 y 4).

En el diagrama de la Figura 10 se presenta el procedimiento seguido desde la toma de muestras, con la metodología empleada en cada caso. Excepto aquellos en los que se indique expresamente, todos los análisis se llevaron a cabo en el Centro de I+D en cerdo Ibérico de Zafra o en el Dpto. de Mejora Genética Animal de Madrid, ambos pertenecientes a CSIC-INIA.

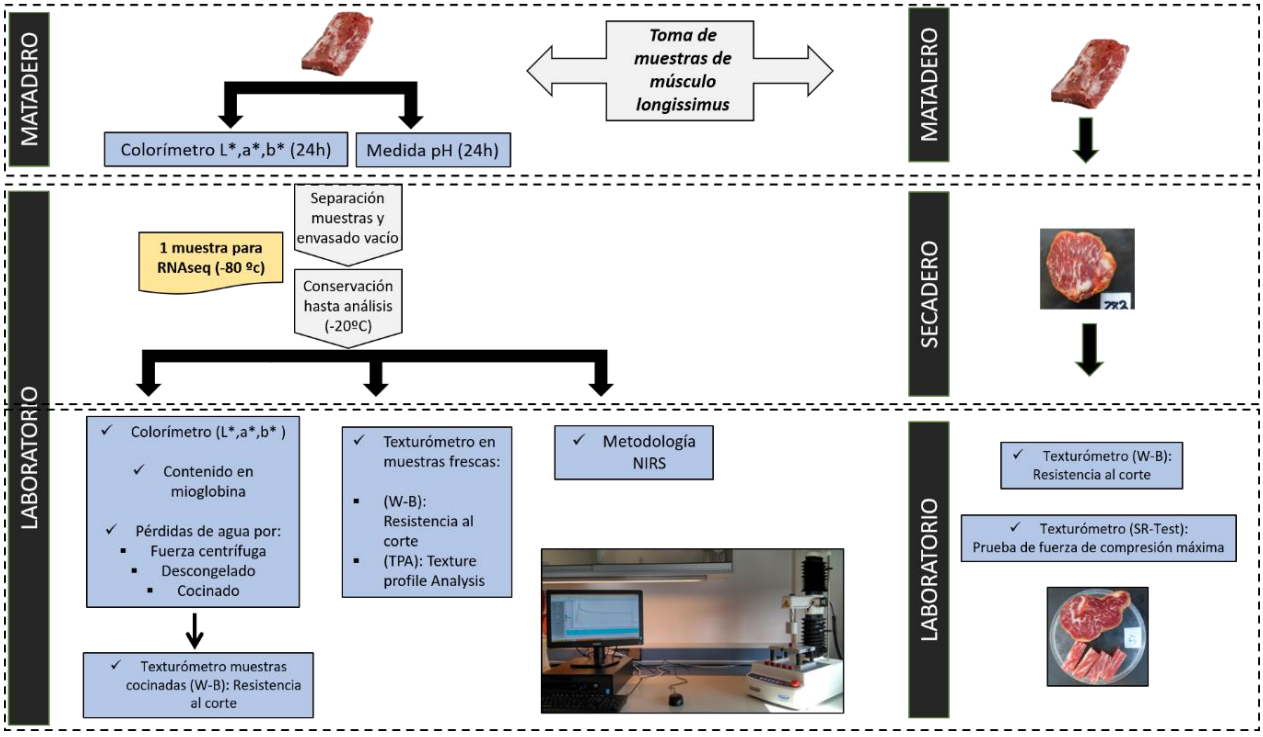


Figura 10. Diagrama de toma de muestras en matadero, secadero y determinaciones de laboratorio.

A continuación, se describe cada uno de estos métodos de manera resumida, mientras que una descripción con mucho más detalle se efectúa en los capítulos de resultados I, II, III y IV de la presente tesis doctoral, que se corresponden respectivamente con los citados experimentos I, II, III y IV.

a) Determinaciones de calidad en muestras de lomo:

- Capacidad de retención de agua: pérdidas de agua por descongelado y por cocinado (Combes et al., 2004) y pérdidas de agua mediante fuerza centrífuga (de acuerdo con Tejerina et al., 2012b).
- Medidas de color: color instrumental mediante colorímetro (L*, a*, b*) y contenido en mioglobina (Hornsey, 1956 con modificaciones de Alberti et al., 2005).
- Determinación de textura mediante texturómetro (Figura 11): resistencia al corte (test Warner-Bratzler; Honikel, 1998), fuerza de compresión máxima (test de stress-relajación; de acuerdo con Morales et al., 2007), dureza, cohesividad, elasticidad y masticabilidad (test TPA; Bourne, 1978).
- Determinación mediante tecnología NIRS de: contenido en mioglobina, pérdidas de agua por fuerza centrífuga, color instrumental (L*, a*, b*), textura resistencia al corte y parámetros TPA, en muestras de lomo enteras y trituradas realizadas en el Departamento de Ciencia y Tecnología Agraria y Alimentaria de la Universidad de Florencia.

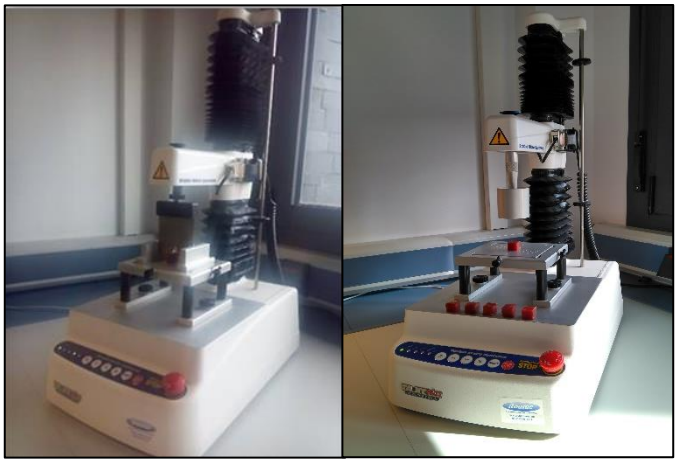


Figura 11. Determinación de textura con texturómetro (Stable Microsystems TA. XT Plus, Godalming, U.K.). Sonda Warner-Bratzler y sonda cilíndrica de compresión (P/50).

b) Extracción de ADN (kit NucleoSpin®, Macherey-Nagel, Düren, Alemania) en muestras de sangre y genotipado de 32 SNPs seleccionados a partir de datos de secuenciación de genoma completo en genes candidatos para calidad de carne de acuerdo con la bibliografía disponible. El genotipado se llevó a cabo utilizando la plataforma de genotipado TaqMan® OpenArray™, (Thermo Fisher Scientific, Waltham, Massachusetts, USA) en el Servicio Veterinario de Genética Molecular (SVGM, UAB, Barcelona).

c) Extracción de ARN de músculo longissimus (kit RiboPure TM of High-Quality total RNA; Ambion, Austin, TX, USA) y control de la calidad. Secuenciación de librerías con fragmentos pareados (*pair-end*) mediante la tecnología Illumina en secuenciadores HiSeq2000 (Illumina, Inc, San Diego, CA, USA), realizado en el Centro Nacional de Análisis Genómico (CNAG-CRG, Barcelona, España) y Novaseq6000 (Illumina, Inc, San Diego, CA, USA) realizado en Novogene (Novogene Company Limited, Cambridge, U.K.).

Los resultados del RNA-seq se validaron mediante RT-qPCR. En un primer paso se sintetizó ADN copia (ADNc) a partir de las muestras de ARN previamente extraídas mediante hexámeros aleatorios. Se diseñaron cebadores para un mínimo de 10 genes por cada uno de los experimentos de RNA-seq (resistencia al corte y contenido en mioglobina) y la cuantificación de la expresión se llevó a cabo utilizando el kit SYBR Green Mix (Roche, Basilea, Suiza) en el aparato LightCycler480 (Roche, Basilea, Suiza). Por cada muestra se cuantificaron tres réplicas técnicas. Los puntos de corte medios se usaron para llevar a cabo los análisis estadísticos.

d) Análisis estadísticos y bioinformáticos:
   - Estima de parámetros genéticos aplicando distintos modelos mixtos multicarácter utilizando el programa VCE-6 (Groeneveld, Kovak, & Mielenz, 2010).
   - Estima de valores mejorantes o aditivos para los caracteres de resistencia al corte y contenido en mioglobina aplicando modelos lineales mixtos unicarácter y utilizando los programas TM (Legarra et al., 2008) y PEST (Groeneveld, Kovak, & Wang, 1999).

- Análisis de asociación entre los caracteres de calidad de carne (del experimento 1, Figura 7) y un panel con 32 SNPs en genes candidatos aplicando un modelo lineal mixto y utilizando el programa Qxpak 4.0 (Pérez-Enciso & Misztal, 2004).
- Determinación de caracteres de calidad (del Experimento 2, Figura 7) mediante la tecnología NIRS. La calibración y validación de los modelos fue desarrollada utilizando regresiones parciales de mínimos cuadrados (PLS) y además se aplicó una validación cruzada interna. El mejor modelo para cada carácter fue evaluado mediante el coeficiente de determinación R^2, el error cuadrático medio (RMSE), la desviación residual predictiva (RPD) y el ratio del rango del error (RER). Para desarrollar las ecuaciones matemáticas se utilizó la región completa del infrarrojo cercano.
- Análisis de datos de secuenciación de transcriptoma (RNA-seq).
  - Análisis de calidad de los datos de secuenciación mediante el programa FastQC (Babraham Bioinformatics, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).
  - Eliminación de adaptadores y filtrado utilizando el programa TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/).
  - Las lecturas filtradas de las muestras de RNA procedentes de cerdos Ibéricos divergentes para resistencia al corte se alinearon y ensamblaron y el análisis de expresión diferencial se analizó mediante el protocolo Tuxedo (Figura 12).
  - Las lecturas filtradas de las muestras de RNA procedentes de cerdos Ibéricos divergentes para contenido en mioglobina se alinearon y ensamblaron y el análisis de expresión diferencial se analizó mediante el protocolo Hisat2-HTseq-counts- DESeq2 (Figura 12).

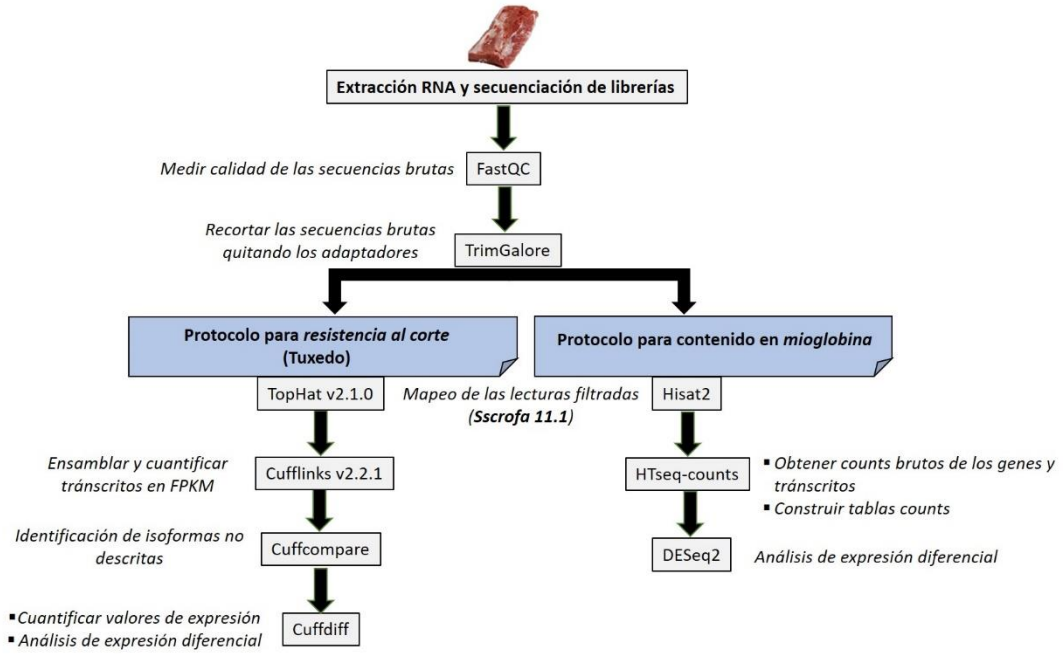


Figura 12. Diagrama de los protocolos de RNA-seq utilizados para el análisis de expresión diferencial entre grupos divergentes para resistencia al corte y contenido en mioglobina.

- Para el análisis funcional de los genes diferencialmente expresados se usó: Babelomics 5 (http://babelomics.bioinfo.cipf.es) para obtener información acerca de las rutas GO. STRING tools v11.0, para las interacciones y clústeres entre las proteínas codificadas por los genes. Además, se usó IPA software (Ingenuity Systems, Qiagen, CA, USA) para las funciones biológicas, redes, rutas génicas y factores reguladores de transcripción.

- Para llevar a cabo los análisis estadísticos de la validación técnica mediante RT-qPCR se usó el programa Genorm para calcular la estabilidad de los genes endógenos *ACTB* y *B2M.* La validación técnica se llevó acabo analizando las correlaciones de Pearson entre los valores de expresión obtenidos del RNA-seq (FPKM o contajes) y la expresión de los datos de genes normalizados de la RT-qPCR. También se calculó el coeficiente de correlación de concordancia (CCC).

RESULTADOS

CAPÍTULO I

Genetic parameter estimation and gene association analyses for meat quality traits in open-air free-range Iberian pigs.

Fernández-Barroso M.A., Silió L., Rodríguez C., Palma-Granados P., López A., Caraballo C., García-Casco J.M., Sánchez-Esquiliche F., Gómez-Carballar F & Muñoz M. 2020. Journal Animal Breeding and Genetics, 137: 581– 598. https://doi.org/10.1111/jbg.12498

Received: 19 December 2019 | Revised: 29 June 2020 | Accepted: 4 July 2020

DOI: 10.1111/jbg.12498

ORIGINAL ARTICLE

Journal of Animal Breeding and Genetics WILEY

Genetic parameter estimation and gene association analyses for meat quality traits in open-air free-range Iberian pigs

Miguel Á. Fernández-Barroso[1,2] | Luis Silió[2] | Carmen Rodríguez[2] | Patricia Palma-Granados[1,2] | Adrián López[2] | Carmen Caraballo[1,2] | Fernando Sánchez-Esquiliche[3] | Fernando Gómez-Carballar[3] | Juan M. García-Casco[1,2] | María Muñoz[1,2]

[1]Centro de I+D en Cerdo Ibérico, INIA, Zafra, Spain

[2]Departamento de Mejora Genética Animal, INIA, Madrid, Spain

[3]Sánchez Romero Carvajal, SRC, Huelva, Spain

Correspondence
Miguel Ángel Fernández-Barroso, Centro de I+D en Cerdo Ibérico, INIA, Zafra, Spain. Departamento de Mejora Genética Animal, INIA, Madrid, Spain.
Email: fernandez.miguel@inia.es

Funding information
Centre for Industrial Technological Development, Grant/Award Number: IDI-20171141, CON15-078 and CON17-125 (INIA-SRC)

Abstract

Meat quality of Iberian pigs is defined by the combination of their genetic characteristics and the particular production system. To carry out a genetic analysis of the main meat quality traits, we estimated their heritabilities, genetic correlations and the association effects of 32 selected SNPs of 12 candidate genes. A total of ten traits were measured in *longissimus dorsi* samples from 1,199 Iberian pigs fattened in the traditional free-range system: water holding capacity (thawing, cooking and centrifuge force water losses), instrumental colour (lightness L*, redness a* and yellowness b*), myoglobin content, shear force on cooked meat, and shear force and maximum compression force on dry-cured loin. Estimated heritability values were low to moderate (0.01 to 0.43) being the lowest for L* and the highest for cooking loss. Strong genetic correlations between water holding capacity traits (0.93 to 0.96) and between myoglobin content and a* (0.94) were observed. The association analyses revealed 19 SNPs significantly associated with different traits. Consistent and strong effects were observed between *PRKAG3* SNPs (*rs319678464G > C* and *rs330427832C > T*), *MYH3_rs81437544T > C*, *CASP3_rs319658214G > T* and *CTSL_rs332171512A > G* and water losses. Also for *CAPN1_rs81358667*G > A and *CASP3_rs319658214G > T* and shear force. The SNPs mapping on *PRKAG3* showed the highest effects on Minolta colour traits. Genotyping of these SNPs could be useful for the selection of Iberian young boars with similar estimated breeding values for productive traits.

KEYWORDS

breeding programme, heritability, Iberian pig, meat quality, SNP

1 | INTRODUCTION

Pigs belonging to the Iberian breed are characterized by having a high adipogenic potential, voracious appetite and high organoleptic meat quality parameters compared to most common breeds in Europe. These features are determined by the combination of their genetic characteristics and its traditional production system. This system is based on an open-air free-range finish-fattening period named *Montanera,* with ad libitum intake of grass and acorns, which reinforces the accretion of subcutaneous and intramuscular fat, with a high content of monounsaturated fatty acids (Lopez-Bote, 1998) and antioxidants (Rey, Daza, López-Carrasco, & López-Bote, 2006).

Most of the porcine breeding schemes in commercial breeds are focused on the improvement of production and reproduction traits such as growth rate, average daily gain, lean composition, number of piglets born alive and/or farrowing rate. The success of these breeding programmes has been well proven (Hill, 2008). However, so far there are not many breeding programmes implemented in the Iberian pig breed. Previously, several breeding programmes have proposed to improve production characteristics such as yield of premium cuts (ham, shoulders and loins) on carcass without a detriment of meat quality traits (Garcia-Casco, Muñoz, Silió, & Rodríguez-Valdovinos, 2014; Silió, 2000). Currently, the objective of porcine sector should be focused on elaborate high-quality meat products without a detriment of the production characteristics.

Both intramuscular fat content and fatty acid composition have been proposed to be included in different Iberian breeding schemes (García-Casco et al., 2014; Ibáñez-Escriche, Magallón, Gonzalez, Tejeda, & Noguera, 2016; Muñoz, Sánchez-Esquiliche, et al., 2018) since they are heritable and it is well known that they are determinants of meat quality traits such as tenderness, juiciness and flavour among others, which are directly related with the consumer acceptance.

However, other quality traits such as tenderness, water holding capacity, pH and colour (Davoli et al., 2017) are also suitable for being included in breeding programmes, especially due to a recent increase in the consumption of Iberian pig meat. Most of the studies analysing meat quality traits in Iberian pigs fattened under the *Montanera* system are focused on checking the effect of differential treatments, diets or production systems and they used a low number of individuals (Mayoral et al., 1999; Ventanas, Ventanas, Jurado, & Estevez, 2006). Therefore, studies that contemplate the estimation of genetic parameters using a large number of samples should be carried out in order to assess the inclusion of the aforementioned meat quality traits in Iberian pig selection schemes.

The inconveniences of including these traits in traditional breeding schemes are that they can be only measured after slaughter (Van Wijk et al., 2005) are laborious and expensive to measure. A more efficient selection strategy could be achieved through genomic selection, genotyping the animals with high-density panels (Fontanesi & Samorè, 2016). However, their high cost makes the practical implementation infeasible in Iberian pig breeding programmes. One potential approach is to identify a few polymorphisms located in candidate genes with significant validated effects on meat quality traits useful for improving them in the population of interest. In previous studies on different pig breeds and cattle, many candidate genes have been reported to be associated with quality traits in both meat and dry-cured products. Some examples are *PRKAG3* (Ciobanu et al., 2001; Milan et al., 2000), *CAPN1* and *CAPN3* (Gandolfi, Pomponio, et al., 2011), *MYOD (*Lee et al., 2012) or *CTSL* genes (Whipple et al., 1990). The singularity of the breed and its unique productive system justify the identification of polymorphisms on these genes in Iberian pig and the analysis of their effects on meat quality traits.

Therefore, the aims of this study are as follows: a) to determine a set of meat quality traits on *longissimus dorsi* (LD) from a large sample of individuals belonging to a closed purebred Iberian line, b) to estimate genetic parameters of the meat quality traits and c) to design a small panel of SNPs mapped on candidate genes and evaluate their potential effect on the meat quality traits.

2 | MATERIAL AND METHODS

2.1 | Animal material

In the present study, commercial castrated male pigs belonging to an Iberian purebred line closed since 15 years were used. In the year 2012, a breeding programme was started with detailed recording of pedigree and production phenotypes. Animals were fed under a restricted feeding regime up to approximately 100 kg of body weight and managed in semi-extensive system grouped in large outdoor fences. Then, they were fattened in an open-air free-range system (*Montanera*) based on ad libitum intake of acorns and grass on a large farm of 468 ha divided in several fenced paddocks. The animals were grouped into two or three individual fattening batches according to live weight and applying a rotational use of acorn in the fences during the four months of the fattening period. Approximately 260 different pigs per season were managed during five successive years (S1, S2, S3, S4 and S5) from 2014 to 2018, being slaughtered in the same slaughterhouse in 20 different batches, at an approximate age of 17 months and with an average slaughter weight of 165 kg. The combination of fattening and slaughter batches resulted in 39 fattening–slaughter batches. The available pedigree information comprised 2,140 individuals. Growth and slaughter data were recorded, and blood samples were collected from 1,199 individuals. Pigs with measured phenotypes were born from 22 sires and 805 dams, from these individuals, a total of 15 sires and 361 dams correspond to the base population. Animal handling was carried out according to the regulations of the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63/EU about the protection of animals used in research. Protocols were assessed and approved by the INIA Committee of Ethics in Animal Research, which is the named Institutional Animal Care and Use Committee (IACUC) for the INIA.

2.2 | Phenotypic data

Longissimus dorsi samples, with an approximate weight of 700 grams, were removed of one of the loins from 1,199 carcasses. Fresh loin samples were divided in different parts according to the posterior subsequent meat quality analyses. After that, they were vacuum-packed in nylon/polyethylene bags and stored at −20ºC for thawing (TL) and cooking loss (CL), instrumental colour (lightness L*, redness a*, and yellowness b*) and cooked meat shear force (SFF) determinations. Besides, one of the portions was introduced in liquid N_2 during approximately 20 s before storing at −20ºC for posterior myoglobin content (MB) and centrifuge force loss (CFL) determination. The other loin was dry-cured following a traditional process by the manufacturing industry, and the individual traceability of samples was kept. Posterior meat quality analyses were carried out on dry-cured product as well.

Water holding capacity (WHC) was determined on LD by three different methods: CFL was measured according to Tejerina, García-Torres, and Cava (2012). After thawing, approximately 1.5 g of minced sample was weighted and wrapped on a filter papers (FILTER-LAB®, Barcelona, Spain) previously weighed. After that, samples were introduced in tubes and centrifuged at 4,000 rpm for 20 min at 16ºC (Thermo Fisher Scientific, Sorvall ST16R, Waltham, Massachusetts, USA). After removing the sample, the filter paper was reweighed. The percentage of CFL was calculated as the difference between initial weight of filter papers and final weight of wet filters in g water/100 g muscle. Measures were done in duplicate. After thawing, the percentage of TL was calculated as the difference between initial weight of the fresh sample and final weight of the thawed samples and the results are presented as g water/100g muscle. These samples were vacuum-packed again in nylon/polyethylene bags and cooked by immersion at 70°C during 1 hr in a water bath (VWR, Pennsylvania, USA) (Combes, Lepetit, Darche, & Lebas, 2004). The difference of weight before and after cooking was used to calculate the percentage of CL, and the results are presented as g water/100 g muscle.

Texture was determined in the cooked meat portions, measured as SFF by Warner–Bratzler test (Stable Microsystems TA.XT Plus, Godalming, U.K.) (Honikel, 1998). Eight pieces of 3 cm × 1 cm × 1 cm (length, width and thickness) were cut perpendicular to the muscle fibre direction with a Warner–Bratzler blade (HDP/BSW), and the eight repeated measures were averaged. SFF was measured as kg/cm^2.

Colour parameters (L*, a*, b*) were measured in thawed meat using a reflectance colorimeter (Konica Minolta CR-400, New Jersey, USA) in a small steak of 2 cm of thickness separated and oxygenated through contact with the air during 15 min. Myoglobin content (MB) was determined following Horsney (1956) with Alberti et al. (2005) modifications. 2.5g of minced sample was introduced in tubes and homogenized with 0.5 ml H_2O milli-Q, 10 ml acetone and 0.25 ml HCL 37% (Fisher Scientific, Loughborough, Leicestershire, UK) for the heminic pigment extraction, besides, a blank with double content of reagents was prepared. The tubes were stored in darkness for 24h. After 24 hr, the content was filtered and the optical density at 512 and 640 nm was measured in a spectrophotometer UV-VIS (Thermo Fisher Scientific, Genesys 10S, Waltham, Massachusetts, USA). Absorbance was converted to mg myoglobin/g muscle. Measures were done by duplicate. Intramuscular fat content (IMF, %) was determined by near infrared spectroscopy (NIRS) following Solís, de Pedro, Garrido, and García-Olmo (2001).

After the dry-cured process of the other loins, they were vacuum-packed and stored at 4ºC until instrumental texture analysis. Two different measurements were carried out; shear force (SFC) that was measured in the same way as that in cooked samples and the maximum compression force (F0) by stress-relaxation test (SR) following Morales, Guerrero, Serra, and Gou (2007), with slight modifications. Samples were cut into eight cubes of 1 cm^3 and compressed in perpendicular direction to the muscle fibres only one time up to 25% of the original height, with a cylindrical probe of 50 mm diameter (P/50). The eight measures of F0 in kg/cm^2 were averaged for each sample.

2.3 | DNA extraction and genotyping

Genomic DNA was extracted from 200 µl aliquots per sample using the NucleoSpin® Blood Kit, (Macherey-Nagel, Düren, Germany) following manufacturer's recommendations. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to measure the concentration and quality of the DNA extracted. All the DNA samples were diluted to 50 ng/µl.

Whole-genome sequences of 15 Iberian individuals were available for SNP calling. Details about genome re-sequencing, assembly and SNP calling were described in a previous study (Muñoz et al., 2020). A subset of 32 SNPs was considered in this study according to the following criteria: a) they map within one of the 12 candidate genes for meat quality (*CAPN1, CAPN3, CAPNS1, CAPNS2, CASP3, CASP9, CTSB, CTSL, MYH1, MYH3, MYOD* and *PRKAG3*), b) they have a MAF > 0.10 in the 15 animals sequenced and c) they fulfil the requirements set by the Custom TaqMan® Assay Design Tool.

A total of 32 selected SNPs were genotyped using a TaqMan® OpenArray™ Genotyping platform (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Genotyping was carried out in a QuantStudioTM 12 K Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at the Veterinary Molecular Genetics

University (SVGM, UAB, Barcelona, Spain). With this technique, SNPs are genotyped through TaqMan probes fixed to a metal-based array. DNA samples were loaded and amplified on the arrays following manufacturer instructions. Detection of allele-specific signal intensities was performed using OpenArray NT Imager, and the genotypes were called using OpenArray SNP Genotyping analysis software. In addition, SNPs images were visually inspected to avoid any clustering issues.

2.4 | Genetic parameter estimation

The inclusion of all traits in a single analysis was not possible due to the lack of convergence. Therefore, a multitrait animal model for three different trait groups was implemented to estimate genetic parameters:

The first group of traits included texture and WHC (SFF, TL, CL, CFL), the four traits related to colour (L*, a*, b* and MB) were analysed in a second group and, finally, the two texture traits measured in dry-cured loins (SFC, F0) were included in the third group.

In order to obtain additional genetic correlation estimates, three complementary analyses were performed using the same multitrait model and grouping the colour traits (L*, a*, b*) with TL and CL, with CFL and with SFF, respectively.

The following general mixed model was applied for all the multitrait analyses:

$$\begin{pmatrix} y_1 \\ . \\ y_i \end{pmatrix} = \begin{pmatrix} X_1 & 0 & 0 \\ 0 & .. & 0 \\ 0 & 0 & X_i \end{pmatrix} \begin{pmatrix} \beta_1 \\ . \\ \beta_i \end{pmatrix} + \begin{pmatrix} Z_1 & 0 & 0 \\ 0 & .. & 0 \\ 0 & 0 & Z_i \end{pmatrix} \begin{pmatrix} u_1 \\ . \\ u_i \end{pmatrix} + \begin{pmatrix} M_1 & 0 & 0 \\ 0 & .. & 0 \\ 0 & 0 & M_i \end{pmatrix} \begin{pmatrix} m_1 \\ . \\ m_i \end{pmatrix} + \begin{pmatrix} e_1 \\ . \\ e_i \end{pmatrix}$$

Where $\boldsymbol{y_1}$ to $\boldsymbol{y_i}$ represent the vectors of phenotypic values corresponding to each trait, $\boldsymbol{\beta_1}$ to $\boldsymbol{\beta_i}$ are the vectors of systematic effects for the corresponding traits, which include IMF, and the average weight of the two loins for each individual as covariates. The vectors $\boldsymbol{u_1}$ to $\boldsymbol{u_i}$ are the random additive genetic effects which have a covariance equal to $\boldsymbol{A} \otimes \begin{pmatrix} \sigma^2_{u1} & \cdots & \sigma_{u1i} \\ \cdots & \cdots & \cdots \\ \sigma_{ui1} & \cdots & \sigma_{ui} \end{pmatrix}$ where $\otimes$ is the Kronecker product and $\boldsymbol{A}$ the numerator relationship matrix, $\boldsymbol{m_1}$ to $\boldsymbol{m_i}$ are the vectors of the random fattening-slaughter batch environmental effects (39 levels), and $\boldsymbol{e_1}$ to $\boldsymbol{e_i}$ are the residual effects. Matrices $\boldsymbol{X_1}$ to $\boldsymbol{X_i}$, $\boldsymbol{Z_1}$ to $\boldsymbol{Z_i}$ and $\boldsymbol{M_1}$ to $\boldsymbol{Mi}$ are the incidence matrices that associate, respectively, the elements of $\boldsymbol{\beta_1}$ to $\boldsymbol{\beta_i}$, $\boldsymbol{u_1}$ to $\boldsymbol{u_i}$ and $\boldsymbol{m_1}$ to $\boldsymbol{m_i}$, with the records in $\boldsymbol{y_1}$ to $\boldsymbol{y_i}$. Variance and covariance components were obtained using VCE-6 program (Groeneveld, Kovak, & Mielenz, 2010).

2.5 | SNP Association Analysis

The effects of the SNPs were independently tested using the following univariate animal model, analogous to those used in the estimation of genetics parameters:

$$\boldsymbol{y = X\beta + Zu + Mm + e}$$

Where $\boldsymbol{y}$ is the vector of phenotypic values corresponding to each studied trait, $\boldsymbol{\beta}$ represents the systematic effects including the same covariates than in genetic parameter estimation and the corresponding SNP effect which takes values of 1 or −1 when the animal was homozygous or 0 if the animal was heterozygous. $\boldsymbol{u}$ is the vector of the remaining polygenic effects distributed as $N(0, \boldsymbol{A}\sigma^2_u)$, where $\boldsymbol{A}$ is the numerator of kinship matrix which allows the adjustment of the data taking into account the pedigree information, $\boldsymbol{m}$ is the vector of the random fattening–slaughter batches effects (39 levels), and $\boldsymbol{e}$ the vector including the residual effects. $\boldsymbol{X}$, $\boldsymbol{Z}$ and $\boldsymbol{M}$ are the incidence matrices. All association analyses were carried out with *Qxpak 4.0 software* (Pérez-Enciso & Misztal, 2004). The effective numbers of markers and traits were estimated according to Nyholt (2004) using the alternative formula proposed by Moskvina and Schmidt (2008). The false discovery rate (FDR) was controlled using the procedure of Storey, Bass, Dabney, and Robinson (2019) based on the distribution of nominal *p-values* resulting from the multiple LR tests setting a threshold *q-value* of 0.05. Intragenic linkage disequilibrium was assessed using *Haploview software v4.2* (J. C. Barrett, Fry, Maller, & Daly, 2005) in the following genes with more than one SNP genotyped: *CAPN1, CAPN3, CTSB, CAPNS2, CTSL, CAPNS1, CASP3, CASP9, MYH3, MYOD* and *PRKAG3*.

2.6 | Expression analyses of *PRKAG3* and *CAPN1* genes

A total of 40 loin samples were collected at slaughter, frozen in liquid nitrogen and stored at −80ºC. Total RNA was extracted with Ribopure High Quality total RNA kit (Ambion, Austin, TX, USA). The cDNA was synthesized from random hexamers with the SuperScript™ II Reverse Transcriptase (Invitrogen, Life Technologies, Paisley, UK). Primer pairs used for quantification were designed using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) from the available porcine sequences stored in the NCBI database for

CAPN1 (GenBank accession number NM_001348784.2) and *PRKAG3* gene (NM_214077.1). Table S1 shows primer sequences. Standard PCRs on cDNA were performed to check amplicon sizes. Quantification was carried out using SYBR Green Mix (Roche, Basel, Switzerland) in a LightCycler® 480 (Roche, Basel, Switzerland) following standard procedures, and data were analysed with LightCycler® 480 SW1.5 software (Roche, Basel, Switzerland). Three replicates were run for each sample, and the specific amplification of the genes was confirmed with their dissociation curves. PCR efficiency was estimated for each gene using the standard curve constructed using four points of cDNA serial dilution. Resulting mean Cp values were used to carry out the statistical analyses. The stability of *B2M* and *ACTB* endogenous genes were tested using GeNorm software (Vandesompele et al., 2002), and both genes were used to normalize the data through their normalization factors, which are the geometric means of the two reference gene quantities. Relative quantities were obtained dividing by these normalization factors.

The relative measures of *CAPN1* and *PRKAG3* gene expression were analysed by analysis of variance (ANOVA) using the following linear model with two fixed effects:

$$y = \text{genotype} + \text{season} + e$$

where **y** is the expression measures of *CAPN1* and *PRKAG3* genes in each individual, **genotype** is the genotype of the analysed polymorphism (being AA, AG and GG for *CAPN1_rs81358667G > A*) and GG, GC and CC for *PRKAG3_rs319678464G > C*) and **season** (three levels), the year in which the samples were collected. Analyses were carried out with *lm* and *ANOVA* functions and *dplyr, tydyr and lsmeans* R libraries in R studio software (Team., 2015).

3 | RESULTS

3.1 | Phenotypic data, heritability and genetic correlation estimates

A summary of the number of observations and main statistical parameters on the recorded traits is shown in Table 1.

Genetic parameters for water losses and shear force on cooked meat are shown in Table 2. The estimated values of heritability (h^2) (diagonal of table) ranged from 0.19 (CFL, TL) to 0.43 (CL) while genetic correlations above the diagonal were high and positive, ranging from 0.71 to 0.96. Genetic correlation between TL and CL was high (0.93), and genetic correlations between CFL and the other two water loss traits were almost one, suggesting strong common genetic effects for these traits. High genetic correlations observed between SFF and all the water loss traits point out that selecting for less water loss will result in more tender meat.

Table 3 shows genetic parameters for colour traits. The estimated values of heritability ranged from 0.01 (L*) to 0.25 (a*). Genetic correlations between MB and redness (a*) and yellowness (b*) were very high and positive, (0.94); this result suggests a common genetic basis for a* and myoglobin content. Besides, high and positive genetic correlation was observed between a* and b* (0.98). Although L* parameter showed moderate and positive genetic correlations with the other colour traits, these values were not different from zero since their standard errors were high.

A complementary multitrait analysis was carried out to estimate genetic correlations between Minolta colour parameters with tenderness and water losses (Table S2). While the genetic correlations with tenderness were not significantly different from zero, since the estimated standard errors were high, a moderate to high and positive genetic correlation was observed with the three water loss traits.

Genetic parameters were also estimated for texture traits measured in dry-cured loins. While heritability estimates were almost zero (0.03) for both SFC and F0, their genetic correlation was high (0.87 ± 0.29), but again, the standard error was high.

3.2 | SNP genotyping and association analysis

Table 4 contains the 32 selected SNPs located in 12 candidate genes for meat quality, which were segregating in the whole-genome sequences of 15 Iberian individuals. All the SNPs were previously described in the release 50 of SNPdb (www.ncbi.nlm.nih.gov/snp/?term=txid9823). Although 17 out of the 32 SNPs map in the coding sequence, most of them were synonymous (12) and the remaining five SNPs cause an aminoacid change in its corresponding protein sequence. The remaining SNPs were located in intronic sequences (10) and in putative regulatory regions (five).

All the SNP except one (*PRKAG3_rs343733804G > A*) were successfully genotyped in a total of 1,199 castrated males (Table S3). Four SNPs, three located in *MYH1* and one in *MYH3* genes were monomorphic. The minor allelic frequencies (MAFs) of the remaining SNPs ranged from 0.06 (*CAPN3_rs323177300C > T*) to 0.47 (*CTSL_rs332171512A > G*). Nine SNPs showed intermediate frequencies (0.4 < MAF<0.5).

Figure 1 shows the linkage disequilibrium between SNPs mapped in the same chromosome. Strong intragenic linkage disequilibrium was observed between the SNPs in some of the analysed genes. A total of 13 SNPs had a r^2 higher than 0.95 and showed a complete linkage disequilibrium:

Two of those mapped in *CAPNS1* (*rs329615471A > C, rs346167312C > G,* Figure 1b), two in *CASP9* (rs324307617C > T, *rs346077419G > A*, Figure 1b)

TABLE 1 Phenotypic data recorded from *longissimus dorsi* muscle of heavy Iberian pigs fattened in a free-range system

Trait	*N*	Mean	*SD*	CV	Max	Min
Fresh/cooked samples						
Shear Force (kg/cm^2)	950	4.33	1.10	25.43	7.52	1.96
Thawing Loss (%)	950	9.45	3.55	37.51	18.11	1.54
Cooking Loss (%)	950	24.11	3.04	12.61	31.79	15.88
CFL (%)	828	32.57	3.48	10.68	41.69	22.49
L*	950	40.06	2.78	6.95	46.88	32.92
a*	950	10.19	1.12	10.97	13.07	7.27
b*	950	6.43	0.99	15.37	9.08	3.83
Myoglobin (mg/ g muscle)	828	1.77	0.31	17.54	2.56	1.02
Intramuscular fat (%)	1,199	4.90	1.72	35.15	9.80	1.70
Loin Weight	1,199	1.56	0.16	11.10	1.99	1.13
Dry-cured samples						
W-B Shear Force (kg/cm^2)	745	7.47	2.03	27.21	12.97	2.31
SR Test - F0 (kg/cm^2)	745	5.40	1.56	28.81	9.90	1.70

Abbreviation: *N*, number of samples; *SD*, standard deviation; CV, coefficient of variation; Max, maximum value; Min, minimum value; CFL, centrifuge force water loss, Minolta parameters, L* lightness, a*, redness, b*, yellowness; SR, stress-relaxation test; F0, maximum compression force.

TABLE 2 Heritability (diagonal) and genetic correlations (above diagonal) estimates of shear force for cooked meat, thawing (%), cooking (%) and centrifuge force losses (%). Standard errors between brackets

	Shear Force	Thawing Loss	Cooking Loss	Centrifuge Force Loss
Shear Force	0.25 (0.06)	0.71 (0.22)	0.91 (0.10)	0.77 (0.22)
Thawing Loss		0.19 (0.07)	0.93 (0.06)	0.95 (0.06)
Cooking Loss			0.43 (0.10)	0.96 (0.06)
Centrifuge Force Loss				0.19 (0.06)

TABLE 3 Heritability (diagonal) and genetic correlations (above diagonal) estimates of Minolta instrumental colour parameters (L*, a* and b*) and myoglobin content. Standard errors between brackets

	L	a*	b*	Myoglobin content
L*	0.01 (0.02)	0.45 (0.71)	0.59 (0.53)	0.32 (0.68)
a*		0.25 (0.09)	0.98 (0.04)	0.94 (0.07)
b*			0.12 (0.06)	0.94 (0.11)
Myoglobin content				0.15 (0.07)

four in *MYH3* (*rs330483504C > A*, *rs81437544T > C*, *rs81211437A > G*, *rs341130038A > C*, Figure 1d), three in *CSTB* (*rs81210988G > A*, *rs344637144G > C*, *rs342116151G > A*, Figure 1e) and the two SNPs of *PRKAG3* genes (*rs319678464G > C*, *rs330427832C > T*, Figure 1f). Consequently, the effective number of independent markers was equal to 13.07. According to the correlations between several of the analysed traits, the effective number of independent traits was 4.75.

Association analyses were carried out between the 27 SNPs segregating in the population and the meat quality traits. All the significant SNPs with a nominal significance (*p-value* < 0.05) are shown in Tables S4 to S7. In addition, significant SNPs satisfying the *q-value* threshold (*p-value* < .05 and *q-value* < 0.05) are shown in the Tables 5 and 6.

Table 5 shows the SNPs significantly associated with tenderness and water losses. The most significant effect for tenderness (SFF) was observed for *CAPN1_rs81358667G > A*,

with an additive effect equal to −0.24 ± 0.05, and *CAPN1_rs81358636G > A* ($a = -0.23 \pm 0.06$), being the G allele in both SNPs associated with a tenderer meat. Significant effects of two SNPs located on *CAPNS2* ($a = 0.22 \pm 0.06$) and *CASP3* ($a = -0.25 \pm 0.06$) were also observed.

The two SNPs with the most relevant effects on the different water loss traits mapped on the *PRKAG3* gene (Table 5), both SNPs (*PRKAG3_rs319678464G > C* and *PRKAG3_rs330427832C > T*) are in strong linkage disequilibrium (Figure 1f) having similar magnitude effects and, therefore, these effects are cofounded. The observed SNP effects show a decrease of approximately a 9%, 4% and 3% of the mean of thawing, cooking and centrifuge water losses, respectively.

On the other hand, different SNP effects were observed for the three water loss traits: *CASP3_rs319658214G > T* significantly affects thawing and cooking losses and *CTSL_rs321623592A > T* affects thawing and centrifuge force losses. Specific effects of *CAPNS1_rs329615471A > C* and *CASP9_rs346188168G > A* were also observed on thawing loss.

Furthermore, effects of the three SNPs mapped to the *MYH3* gene on cooking loss and *CAPN1_rs81358667G > A*, *CTSL_rs332171512A > G* and *MYOD_rs345162462T > C* on centrifuge force loss were observed.

Table 6 shows SNPs affecting different colour traits. Both *PRKAG3_rs319678464G > C* and

TABLE 4 Genome location of the 32 SNPs used in the panel, identification in SNPdb, variant effect and their corresponding alleles

Gene	Chromosome	Position (*Ssc10.2*) (Mb)	Position (*Ssc11.1*) (Mb)	SNPdb	Variant effect	Reference	Alternative
CAPN3	SSC1	143,858,984	128,966,764	rs323177300	Missense	C	T
CAPN1	SSC2	6,129,746	6,997,711	rs81358636	3' UTR	G	A
CAPN1	SSC2	6,130,068	6,998,033	rs196954097	3' UTR	G	C
CAPN1	SSC2	6,152,143	7,020,105	rs81358667	Synonymous	G	A
MYOD1	SSC2	44,487,341	41,426,838	rs345162462	Upstream	T	C
CAPNS2	SSC6	27,482,795	30,015,490	rs323301722	3' UTR	C	T
CAPNS1	SSC6	40,916,407	45,514,212	rs329615471	Synonymous	A	C
CAPNS1	SSC6	40,917,052	45,514,857	rs346167312	Intron	C	G
CAPNS1	SSC6	40,918,135	45,515,940	rs789976262	Intron	T	A
CASP9	SSC6	69,091,573	74,710,974	rs346188168	Synonymous	G	A
CASP9	SSC6	69,096,386	74,715,785	rs324307617	Intron	C	T
CASP9	SSC6	69,111,271	74,729,887	rs346077419	Missense	G	A
CTSL	SSC10	31,909,146	27,670,246	rs332171512	Synonymous	A	G
CTSL	SSC10	31,912,041	27,673,190	rs321623592	Intron variant	A	T
CTSL	SSC10	31,912,480	27,673,629	rs340013902	Missense	T	C
MYH1	SSC12	57,967,316	55,242,970	rs339679793	Synonymous	C	T
MYH1	SSC12	57,968,019	55,242,267	rs695620698	Synonymous	A	G
MYH1	SSC12	57,970,468	55,239,818	rs341385020	Synonymous	C	T
MYH3	SSC12	58,116,739	55,367,462	rs81437544	Intron variant	T	C
MYH3	SSC12	58,121,269	55,362,939	rs330483504	Synonymous	C	A
MYH3	SSC12	58,123,322	55,360,886	rs341130038	Intron variant	A	C
MYH3	SSC12	58,127,472	55,356,737	rs333004392	Missense	G	T
MYH3	SSC12	58,130,567	55,353,643	rs81211437	Synonymous	A	G
CTSB	SSC14	16,205,459	15,016,754	rs326566953	Intron	A	G
CTSB	SSC14	16,208,947	15,020,238	rs81210988	Synonymous	G	A
CTSB	SSC14	16,211,464	15,021,094	rs344637144	Intron	G	C
CTSB	SSC14	16,212,122	15,022,755	rs342116151	Synonymous	G	A
CASP3	SSC15	52,293,396	45,751,305	rs321490445	Intron	C	A
CASP3	SSC15	52,305,243	-	rs319658214	Downstream	G	T
PRKAG3	SSC15	133,802,136	120,865,227	rs343733804	Missense	G	A
PRKAG3	SSC15	133,805,574	120,861,791	rs319678464	Intron	G	C
PRKAG3	SSC15	133,806,594	120,860,772	rs330427832	Synonymous	C	T

PRKAG3_rs330427832C > T have significant effects on a* and b* Minolta parameters and nominal effects on myoglobin content (Table S6). Besides, *CTSL_rs321623592A > T* showed a relevant effect on L* ($a = 0.43 \pm 0.14$) and also affects thawing and centrifuge force losses, supporting a common genetic background among water loss and meat colour traits, previously observed in genetic parameter estimation (Table S2). The two SNPs in the *CASP9* gene are in strong linkage disequilibrium (Figure 1b) and had the most significant effects on myoglobin ($a = 0.05 \pm 0.02$).

Although none of the SNPs showed significant effects on rheological traits of dry-cured samples, remarkable effects with nominal significance of *CAPNS1_rs329615471A > C* were observed both in SFC ($a = 0.49 \pm 0.21$) and F0 (0.45 ± 0.17) (Table S7). In addition, nominal effects of *PRKAG3_rs330427832C > T* and *CAPN1_rs196954097G > C* on SFC and F0, respectively, were also observed.

It should be noted that the significant effects of several SNPs observed here should be taken cautiously since a very low frequency for some of their genotypes or even their absence was observed. This is the case of *CAPN1_rs81358636G > A* (GG = 0.02), *CAPN1_rs196954097G > C* (GG = 0.00), *CAPNS2_rs323301722C > T* (TT = 0.04), *CAPNS1_rs329615471A > C* (CC = 0.00), *CASP9_rs346188168G > A* (AA = 0.01), *CASP9_rs324307617C > T*

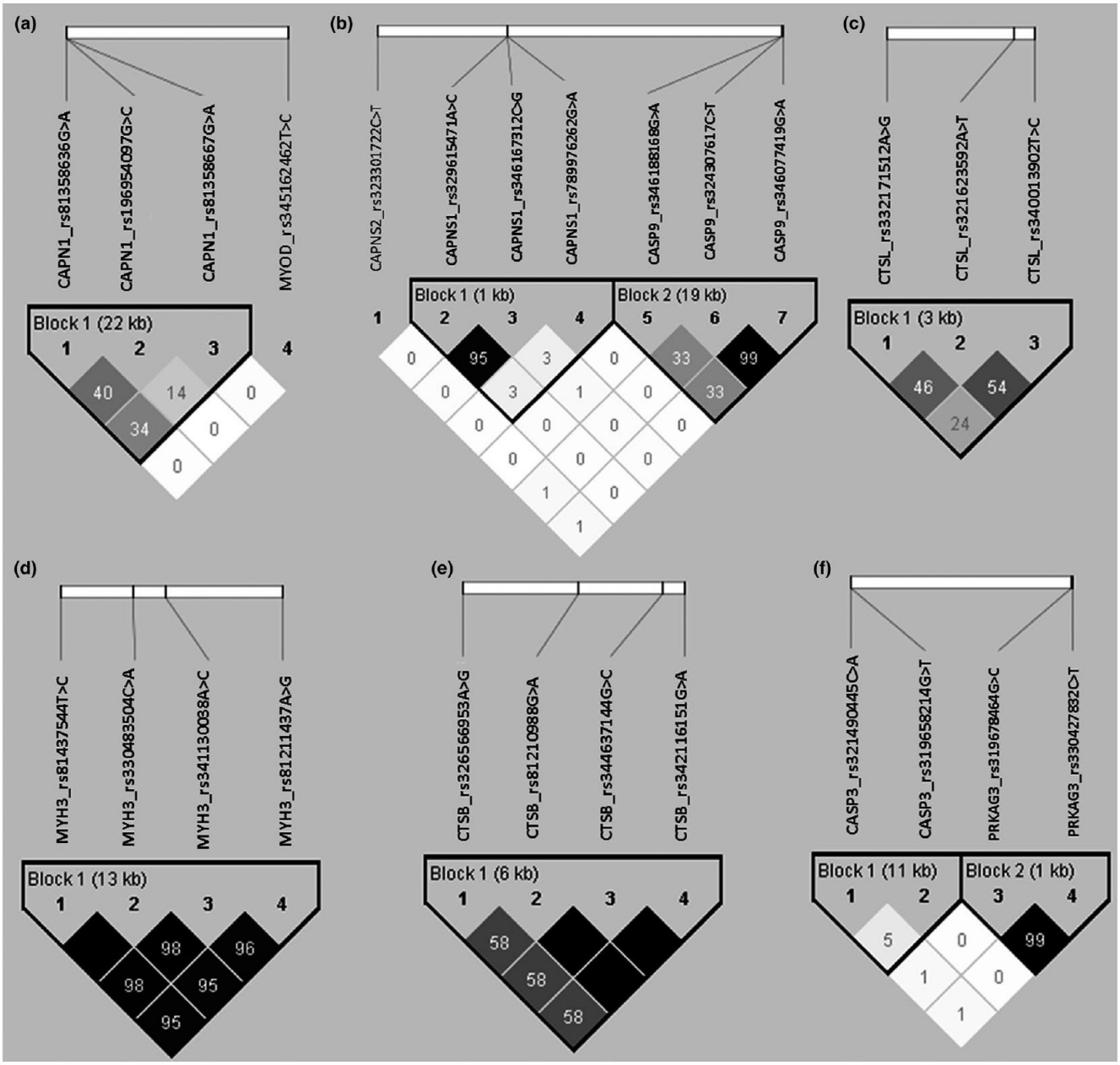


FIGURE 1 Haplotype diagram of the SNPs located in the a) *CAPN1* and *MYOD* genes (SSC2), b) *CAPNS1*, *CAPNS2* and *CASP9* genes (SSC6), c) *CTSL* gene (SSC10), d) *MYH3* gene (SSC12), e) *CTSB* gene (SSC14) and f) *CASP3* and *PRKAG3* genes (SSC15)

TABLE 5 SNPs of candidate genes with significant effects on shear force, centrifuge force loss, thawing and cooking loss

SNP	a (SE)	PM	LR	p-value	q-value
Shear Force (kg/cm^2)					
CAPN1_rs81358636G > A	−0.23(0.06)	−5.31	11.24	7.99×10^{-4}	0.005
CAPN1_rs81358667G > A	−0.24 (0.05)	−5.54	20.89	4.86×10^{-6}	1.31×10^{-4}
CAPNS2_rs323301722C > T	0.22 (0.06)	5.08	11.39	7.39×10^{-4}	0.005
CASP3_rs319658214G > T	−0.25 (0.06)	−5.77	17.88	2.35×10^{-5}	3.17×10^{-4}
Thawing Loss (%)					
CAPNS1_rs329615471A > C	0.57 (0.24)	6.03	6.38	.010	0.040
CAPNS2_rs323301722C > T	0.49 (0.17)	5.19	8.66	.003	0.020
CASP3_rs319658214G > T	−0.51 (0.16)	−5.40	11.84	5.81×10^{-4}	0.004
CASP9_rs346188168G > A	0.83 (0.24)	8.78	11.99	5.35×10^{-4}	0.004
CTSL_rs321623592A > T	0.38 (0.15)	4.02	6.82	.009	0.040
PRKAG3_rs319678464G > C	−0.88 (0.16)	−9.31	28.61	8.86×10^{-8}	1.29×10^{-6}
PRKAG3_rs330427832C > T	−0.88 (0.16)	−9.31	28.46	9.55×10^{-8}	1.29×10^{-6}
Cooking Loss (%)					
CAPNS2_rs323301722C > T	0.56 (0.17)	2.32	10.04	.002	0.006
CASP3_rs319658214G > T	−0.49 (0.16)	−2.03	10.00	.002	0.006
MYH3_rs330483504C > A	0.50 (0.15)	2.07	11.74	6.11×10^{-4}	0.004
MYH3_rs341130038A > C	0.49 (0.15)	2.03	11.40	7.34×10^{-4}	0.004
MYH3_rs81211437A > G	0.47 (0.15)	1.95	9.22	.002	0.008
MYH3_rs81437544T > C	−0.50 (0.15)	2.07	11.67	6.35×10^{-4}	0.004
PRKAG3_rs319678464G > C	−1.02 (0.16)	−4.23	41.91	9.55×10^{-11}	1.29×10^{-9}
PRKAG3_rs330427832C > T	−1.04 (0.16)	−4.31	42.09	8.72×10^{-11}	1.29×10^{-9}
Centrifuge force loss (%)					
CAPN1_rs81358667G > A	−0.45 (0.17)	−1.38	6.93	.008	0.040
CAPNS1_rs789976262G > A	0.61 (0.23)	1.87	7.16	.007	0.040
CTSL_rs321623592A > T	0.46 (0.17)	1.41	7.04	.008	0.040
CTSL_rs332171512A > G	0.59 (0.17)	1.81	11.90	5.62×10^{-4}	0.005
MYOD_rs345162462T > C	−0.46 (0.18)	−1.41	6.31	.010	0.040
PRKAG3_rs319678464G > C	−0.83 (0.19)	−2.55	19.43	1.04×10^{-5}	2.81×10^{-4}
PRKAG3_rs330427832C > T	−0.78 (0.19)	−2.39	17.99	2.22×10^{-5}	3.00×10^{-4}

Abbreviations: a, additive effect; LR, likelihood ratio test values; PM, additive effect expressed as a percentage of the mean of the trait; SE, standard error.

(CC = 0.05) and *CASP9_rs346077419G > A* (GG = 0.05) (Table S3).

3.3 | Expression analyses of *PRKAG3* y *CAPN1* genes

The expression of *CAPN1* and *PRKAG3* genes was assessed in loin samples according to the genotypes of the *CAPN1_rs81358667G > A* and *PRKAG3_rs319678464G > C*, respectively, with the aim of analysing a possible association between the expression of these genes and their corresponding genotype. The number of samples per genotype was even (13 homozygous of each type and 14 heterozygous for both SNPs). Finally, significant expression differences of genes depending on the genotype were not observed, neither for *CAPN1_rs81358667G > A* (*p-value* = 0.132) nor for *PRKAG3_rs319678464G > C* (*p-value* = .225) (Figure 2).

4 | DISCUSSION

Iberian purebred pigs fattening in an open-air free-range system represent 10% of the Iberian porcine sector in Spain, since most of the production is focused on Duroc × Iberian crossbred pigs under intensive or semi-extensive production systems (Nieto et al., 2019). While these crossbred animals are slaughtered around 10–12 months of age, the animals of the current study, belonging to the most representative genetic variety, *Retinto* strain, were slaughtered at approximately 17 months of age. This

TABLE 6 SNPs of candidate genes with significant effects on Minolta colour parameters (L*, a* and b*) and myoglobin content

SNP	a (SE)	PM	LR	*p*-value	q-value
*L**					
CTSL_rs321623592A > T	0.43 (0.14)	1.07	9.39	.002	0.030
MYOD_rs345162462T > C	−0.44 (0.15)	−1.10	9.14	.003	0.030
*a**					
PRKAG3_rs319678464G > C	−0.34(0.07)	−3.34	23.27	1.4×10^{-6}	1.89×10^{-5}
PRKAG3_rs330427832C > T	−0.34(0.07)	−3.34	23.66	1.15×10^{-6}	1.89×10^{-5}
*b**					
CAPNS1_rs789976262G > A	0.23 (0.06)	3.58	14.50	1.4×10^{-4}	0.001
CASP3_rs321490445C > A	0.19 (0.08)	2.95	6.96	.008	0.040
CASP9_rs346188168G > A	0.22 (0.08)	3.42	6.99	.008	0.040
PRKAG3_rs319678464G > C	−0.27 (0.05)	−4.20	26.03	3.37×10^{-7}	7.64×10^{-6}
PRKAG3_rs330427832C > T	−0.27 (0.05)	−4.20	25.03	5.66×10^{-7}	7.64×10^{-6}
Myoglobin content (mg/g)					
CASP9_rs324307617C > T	0.05 (0.02)	2.82	8.96	.003	0.040
CASP9_rs346077419G > A	0.05 (0.02)	2.82	8.54	.003	0.040

Abbreviations: a, additive effect; LR, likelihood ratio test values; PM, additive effect expressed as a percentage of the mean of the trait; SE, standard error.

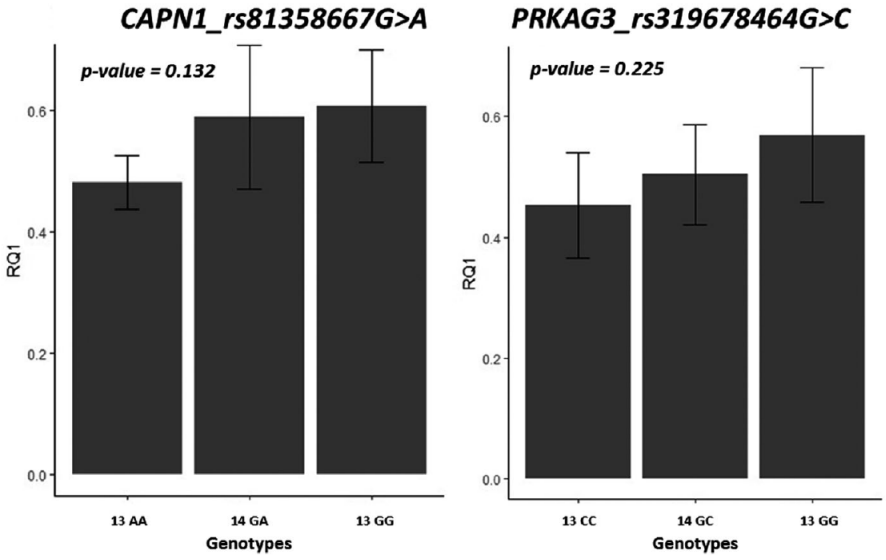


FIGURE 2 *CAPN1* and *PRKAG3* gene expression using RT-qPCR

market structure makes that most of the previous studies have focused on the analyses of meat quality traits in Duroc × Iberian pigs. Therefore, to our knowledge, this is the first study with the highest number of records of meat quality traits measured in Iberian purebred pigs fattening in an open-air free-range system.

4.1 | Phenotypic data

In two studies on Iberian pigs fattening in open-air free-range system, Tejerina et al. (2012) reported similar mean values for SFF, CL and CFL than those founded in the present study; however, TL values were higher (15.6%). Almeida et al. (2018) obtained similar values for SFF and CL. In the same study, they confirmed lower shear force of Iberian pig meat than Large White × Landrace crossbred pigs fed under an intensive system (4.73 and 5.76 kg/cm^2, respectively).

Almeida et al. (2018) also reported higher values for L* (53.33) and similar for a* and b* compared with our results. The lower L* and higher a* values observed are related to a higher content in oxidative muscle fibres in Iberian pigs compared with commercial breeds that are selected for faster growth and have a higher content in glycolytic muscle (Juarez, Clemente, Polvillo, & Molina, 2009 and Weiler, 1995). Pugliese et al. (2005) have proposed that pigs raised outdoors with enough space for doing exercise have less pale and more reddish meats than those reared under intensive production.

Uneven values of MB content were observed in different studies in Iberian pigs. While Estévez, Morcuende, and Cava López (2003) reported similar values than ours for pigs of seven months slaughtered at 85–90 kg live weight, measures of MB in Mayoral et al. (1999) comparing different weights and ages at slaughter were higher at 16 months (2.5 mg/g). Serra et al. (1998) observed lower values (1.25 mg/g) in Iberian pigs from the *Guadyerbas* inbred line (Toro, Rodrigáñez, Silió, & Rodríguez, 2000) reared under commercial intensive management and slaughtered at 100 kg. These differences are related to production systems and slaughter age, since myoglobin content tends to increase during lifetime reaching a maximum peak for older animals (Mayoral et al., 1999).

Iberian dry-cured products have an added value related with cultural and economic factors. One of the main determinants of its quality is the tenderness, but there are not many references reporting analytical texture values in Iberian dry-cured products. Ramírez and Cava (2007) reported values ranging from 0.84 to 0.53 kg/cm^2 and 22.44 to 19.52 kg/cm^2 for SFC and F0, respectively, in Duroc × Iberian crossbred pigs raised in a semi-extensive system. These values were lower than those observed in the current study for SFC and higher for F0. On the other hand, Pateiro, Franco, Carril, and Lorenzo (2015) reported lower values for SFC (3.21 kg/cm^2) and higher for F0 (11.28 kg/cm^2) for dry-cured loin samples from Celta pigs breed feed ad libitum with commercial concentrate in extensive system. This disparity of results could be attributed to differences in sample size and the percentage of compression used for measuring the F0 trait.

4.2 | Heritability and genetic correlations estimates

To our knowledge, there are not any studies reporting genetic parameters for the analysed meat quality traits in Iberian pigs. Studies in other breeds or crosses and genetic parameter estimates are diverse and, in most cases, different from our results.

Most of the heritability estimates reported for shear force and water holding capacity traits (Suzuki et al. (2005), Gjerlaug-Enger, Aass, Odegård, and Vangen (2010), Jung, Kim, Park, Choi, and Park (2011), Miar et al. (2014), Baby et al. (2014) or Lee et al. (2015)) showed moderate-high values (from 0.20 to 0.51); except Suzuki et al. (2005), who reported low values for TL and CL (0.14 and 0.09, respectively). Estimates of genetic correlation between these traits were generally lower (ranging from 0.20 to 0.58) than those showed in Table 2, and some of them were close to zero or even negative (Jung et al., 2014). The heritability estimates presented in this study (Table 2) are below moderate values (TL and CFL, even SFF). Only for CL, a moderate value was observed that supports a positive response to the eventual genetic selection for this trait. However, all the genetic correlations are positive, very high and favourable, implying that selection for each on these traits will generally have a positive response in the other traits as well. Meat with lower water loss seems to be tenderer and selection for any type of water loss would result in the improvement of the other meat quality traits as well. It should be noted that some of standard errors of genetic correlation estimates are generally large, that should be due to the sample size.

Regarding the heritability estimates reported for colour traits (Larzul et al. (1997), Newcom et al. (2004), Suzuki et al. (2005), Gjerlaug-Enger et al. (2010), Miar et al. (2014) and Lee et al. (2015)), most of them are higher (from 0.16 to 0.46) than our results. Genetic parameter estimates in open-air free-range systems are strongly influenced by environmental conditions and the values are usually lower than those observed in intensive systems (García-Casco et al., 2014). In our study, fattening-slaughter cohort effects explained between 18% and 33% of the total variance for colour traits. Regarding L*, the heritability estimate was practically zero being the residual effects an 80% of the total variance. We found quite different results for genetic correlations between colour traits by diverse authors. In summary, previous studies have generally found zero or negative correlations between L* and a*, moderate correlations between L* and b* as well between a* and b*, whereas correlations between MB and L* were found to be slightly negative and those between MB and a* were slightly positive. After the analysis of our data, low to moderate estimates of heritability were observed for colour traits (Table 3). Only for redness a* colour the heritability value (0.25) can be useful for improvement purposes, precisely one of the attributes more valued by consumers. Another interesting result is the high genetic correlation (0.94) between myoglobin content and the a* colorimeter parameter. A high Myoglobin content is related with traditional extensive systems (Ventanas, Ventanas, Ruiz, & Estevez, 2005). Minolta colorimeter is a less expensive and easier measure technique than the determination of Myoglobin content, and therefore, a selection towards an increase of a* parameter would also improve the Myoglobin content and a* would be an advisable selection objective. Moreover, the use of Minolta colorimeter for determination of a* could be also studied for its implementation in the slaughterhouse.

Genetic correlation estimates between SFF and WHC with colour traits (Jung et al. (2011), Miar et al. (2014) and Lee et al. (2015)) point to positive correlations ranging from 0.27 to 0.76, except Jung et al. (2011) and Lee et al. (2015) which found negative values for L* and CFL. Table S2 suggests a strong positive genetic correlation between water loss and lightness L* measured in our samples. This result supports that the ability to retain intrinsic water is reduced

on more pale meat, PSE (Pale, Soft and Exudative) (Listrat et al., 2016), although PSE meat is unusual in Iberian pig carcasses. On the other hand, the genetic correlations shown here between WHC and a* and b* (positive, ranging from 0.26 and 0.95), must be taking into account in an eventual breeding programme because an improvement of redness could involve higher water loss.

Regarding rheological characteristics in dry-cured products, there are not any studies reporting genetic parameters on Iberian pigs, so far. In the current study, the heritability estimates for SFC and F0 were practically zero. Most of the observed variance was due to random *fattening-slaughter* batch effects, which explain 37% and 28% of the total variance for SFC and F0, respectively, and the residual variance, which makes up the remaining 59% and 69%. The dry-cured process comprised several steps (salted, marinated, stuffed and dry-cured) and the model does not consider them and, possibly, it could not estimate the genetic component correctly.

4.3 | Association analyses

The genetic uniqueness of the Iberian pig breed has been addressed by different genetic studies. Unlike most of the commercial European breeds, there is a lack of introgression of Asian mitochondrial DNA haplotypes (Alves, Ovilo, Rodriguez, & Silio, 2003) and studies with microsatellite markers clustered Iberian breed away from other commercial breeds (Ollivier, 2009; SanCristobal et al., 2006). A recent study also reported that several of the polymorphisms considered as causative mutations or mapped on candidate genes for different traits (e.g. *ESR1*:*c.669T* > *C*, *KIT*:*g.41488472C* > *T*, *LEPR*:*c.1987C* > *T*, *PPARD*:*g.31281804G* > *A*, *RYR1*:c.1843C > T, *TAS2R4*:*g.8135115A* > *T* and *TYRP1*:*g.209733431A* > *G*) did not segregate in a representative sample of 48 Iberian individuals (Muñoz, Bozzi, et al., 2018). These genetic peculiarities linked to the genetic isolation and adaptation to a harsh environment may have resulted in different SNPs affecting meat quality traits. Therefore, we searched for polymorphism mapped in the targeted candidate genes, which were segregating in the available whole-genome sequences of 15 Iberian individuals, developing in this way a SNP panel for meat quality traits suitable for the Iberian breed.

In the current study, *PRKAG3, CAPN1, CAPNS1, CAPNS2, CASP3, CASP9, CTSL, MYOD* and *MYH3* genes harbour SNPs with significant effects in more than one of the traits analysed. Although there are many studies in other breeds that found SNPs mapping on these candidate genes with effects on the same meat traits, most of the associations between the SNPs and meat quality traits reported in the current study are described for the first time in the Iberian pig breed, so far.

PRKAG3 gene encodes for the Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma 3. This protein affects cell metabolism when the animals are in stress situation (Thornton, Snowden, & Carling, 1998) producing a change on water holding capacity, which in turn is related with the production of Pale, Soft and Exudative meat (PSE meat), affecting final pH and meat colour (Lightness) (Listrat et al., 2016). Milan et al. (2000) reported the substitution *R200Q* in the Hampshire breed caused an increase in muscle glycogen and raised the production of lactate, generating a lower ultimate pH and poorer water holding capacity. Berkshire × Yorkshire pigs with *30T, 52G* and *199I* alleles had lower muscle glycogen content and lower Minolta L* value (Ciobanu et al., 2001). Homozygous *I199I* Large White × Landrace × Duroc crossbred pigs had less water loss and a lower L* value (Otto et al., 2007). Large White and Large White × Landrace populations with *I199V* and *G52S* heterozygotes had lower thawing loss and higher water holding capacity (Chen et al., 2008). Škrlep, Kavar, & Čandek-Potokar (2010) reported effects of *200Q, 200R, 199I* and *199V* alleles on thawing loss and colour traits in commercial pigs and also Škrlep et al. (2012) described significant effects of *I199* on a* and texture parameters of dry-cured hams, where the homozygous genotype was associated with more tender meat. More effects of *T30N* and *I199V* on cooking loss and L* were found by Rohrer, Nonneman, Miller, Zerby, and Moeller (2012). Finally, Nonneman et al. (2013) found significant associations of *rs80936921* and *rs45434456* SNPs with cooking loss in Landrace × Duroc × Yorkshire pigs. From these SNPs reported by other authors, just *T30N* and *G52S* segregated in the sequenced 15 Iberian animals. However, *T30N* had a MAF lower than 0.10 and was not included in the SNP panel and the *G52S* that corresponds to our *PRKAG3_rs343733804G* > *A* failed in the genotyping process. In the current study, intronic and synonymous SNPs mapped on *PRKAG3* gene (*rs319678464G* > *C* and *rs330427832C* > *T*, respectively) that were in complete linkage disequilibrium, significantly affect water loss traits and a* and b* Minolta parameters (Tables 5 and 6), being the G and C alleles associated with less water loss and less redness meat, respectively. Besides suggestive effects of both SNPs on L*, myoglobin content and shear force of dry-cured products were also observed (Tables S6 and S7). Therefore, the results found on these SNPs mapped on *PRKAG3* make them appropriate candidates to be included in a breeding programme for reducing water loss in the referred population.

Proteolysis is a *post-mortem* process involved in the transformation of muscle to meat, being the main determinant of tenderness. The *CAPN1* gene encodes for three protein isoforms (μ-calpain, m-calpain and calpain 3), that constitute the protease system involved in proteolysis. There are consistent evidences that calpains are linked to the tenderization of beef, lamb and pork (Koohmaraie & Geesink, 2006;

Sentandreu, Coulis, & Ouali, 2002). Although there are not studies that report any significant effects of *CAPN1* SNPs either on pig meat tenderness or on water holding capacity, there are several studies in cattle that showed *CAPN1* SNP effects on shear force. In Limousine and Jersey steers, the haplotypes *530I* and *316G* showed higher shear force values (Page et al., 2002). In Charolais breed, Allais et al. (2011) and Aviles et al. (2013) described effects of some SNPs for shear force. In the same direction, Sun et al. (2018) found that *4558A > G* and *4684C > T* SNP*s* were significantly associated with shear force in cooked meat samples of Chinese cattle breed. Regarding colour traits, Gandolfi, Pomponio, et al. (2011) observed effects of *HM535412:g.157T > C* SNP on a* parameter measured on loins of Duroc × (Landrace × Large White) crossbred pigs. The SNP described by Gandolfi, Pomponio, et al. (2011) is segregating in the whole-sequences available for the 15 Iberian pigs; however, this was not included in our SNP panel since we observed another SNP segregating at three base pairs that could interfere in the genotyping process with OpenArray™. Therefore, we decided to genotype *rs81358667G > A* (MAF = 0.33) located within the coding region at 103 bp and with a higher MAF than *HM535412:g.157T > C* in this 15 individuals. The SNP *CAPN1_ rs81358667G > A* showed significant effects on shear force and centrifuge force loss (Table 5). In addition, relevant effects of *CAPN1_ rs81358636G > A* on shear force (Table 5) were observed. Suggestive effects of these SNPs were observed on thawing and cooking losses and for *CAPN1_rs196954097G > C* on L* and maximum compression force of dry-cured products (Tables S5, S6 and S7). However, these two last SNPs presented a very low frequency of the GG genotype (Table S3) and it is likely that the additive effect of the allele has not been correctly estimated. Hence, the association with shear force turns the *rs81358667G > A* in a good candidate to be used for selecting more tender meat in this population.

The complementary expression analyses of *PRKAG3* and *CAPN1* genes on LD muscle according to the genotypes of *CAPN1_rs81358667G > A* and *PRKAG3_rs319678464G > C* SNPs did not show any association between their genotypes and the expression of each gene. This fact does not imply the SNPs are not the causative mutation explaining the effects on the analysed traits and other functional studies such as to analyse the enzymatic activity of the protein according to the different SNP genotypes should be carried out.

CAPNS1 and *CAPNS2* genes encode for the Calpain Small Subunit 1 and 2, respectively. While CAPNS1 protein is very important for maintaining stability of both conventional calpain catalytic subunits (Goll, Thompson, Li, Wei, & Cong, 2003), the physiological role of CAPNS2 protein is unclear (Sorimachi, Hata, & Yasuko, 2011). The porcine *CAPNS1* gene is located on SSC6 and within this region; a QTL affecting water loss traits was reported by de Koning et al. (2001). Besides, Ponsuksili et al. (2008) identified correlated expression between *CAPNS1* gene and drip loss and Gandolfi, Cinar, et al. (2011) reported a suggestive effect of *c.429A > C* on cooking loss in Duroc × Pietrain crossbred. These studies are in agreement with SNP associations showed in the present study for water loss traits. The same SNP described by Gandolfi, Cinar, et al. (2011), here named as *rs329615471A > C*, have relevant effects on TL and *rs789976262G > A* showed significant effects on CFL (Table 5). However, both SNPs presented low frequencies of CC and AA genotypes, respectively (Table S3), making these SNPs not suitable for selection purposes in this population.

CASP3 and *CASP9* genes code for caspases 3 and 9, respectively. Caspases are a family of cysteine aspartate-specific proteases that participates in tenderization with roles in apoptosis and inflammation (Ouali et al., 2006; Sentandreu et al., 2002). Kemp, Sensky, Bardsley, Buttery, and Parr (2010) hypothesized that the process of slaughter and exsanguination could initiate apoptotic pathways and caspase activity may contribute to early *post-mortem* proteolysis and meat tenderization. Actually, this study estimated a negative relationship between CASP3 and CASP9 activity and shear force values measured during the early *post-mortem* period, when caspases are more active. This relation could be associated with meat tenderization. In the current study, significant effects of *CASP3_rs319658214G > T* on tenderness and thawing and cooking losses have been estimated (Table 5). Moreover, effects of *CASP9_rs346188168G > A* SNP on TL and b* and of *CASP9_rs324307617C > T* and *CASP9_rs346077419G > A* SNPs on myoglobin content were also observed (Table 6). Besides, *CASP9_rs346188168G > A* showed a nominal effect on a*. From this set of SNPs, *CASP3_rs319658214G > T* is the most suitable for being used in selection for tenderness, since the other three present a frequency of one of the genotypic class lower than 0.05 (Table S3).

The *CTSL* gene encodes for the Cathepsin L protein, a lysosomal cysteine proteinase that plays a major role in intracellular protein catabolism. Cathepsins also participate in *post-mortem* proteolysis and meat tenderization as other enzymatic proteases such as calpains, calpastatin and caspases. Whipple et al. (1990) reported an association between cathepsin activity and the variation on tenderness in cattle meat samples. Although in the current study any association between *CTSL* SNPs and tenderness was found, the intronic SNP *rs321623592A > T* mapped on SSC10 presented significant associations on thawing, centrifuge force losses and L*. Also *rs332171512A > G* shown a relevant effect on centrifuge force loss (Table 5). The stronger magnitude of this last effect makes *rs332171512A > G* as another good candidate for selection objectives in a breeding programme.

MYOD gene encodes for the nuclear Myogenic Differentiation protein 1. *MYOD* belongs to the Myogenic

Regulatory Factors protein family and regulates muscle cell differentiation and development by inducing cell cycle arrest. Therefore, SNPs within *MYOD* gene could influence muscle fibres type I (oxidative and red muscle) and type II (glycolytic and white muscle), meat production and meat quality (Kim, Choi, Kim, Park, & Hong, 2009; te Pas et al., 1999). Lee et al. (2012) reported a significant association of *1264C > A* SNP with Minolta L* parameter, where the homozygous CC shown lower L* values on a study with Yorkshire and Berkshire pigs on LD muscle. However, there were no effects on WHC traits in the same study. Han et al. (2012) presented a significant effect of the *g.257A > C* on thawing loss of Large White pigs. Those SNPs were not included in the SNP panel since they were not segregating in the available whole-genome sequences. In the current study, we found significant effects of *rs345162462T > C* on centrifuge force loss and L* (Tables 5 and 6), also suggestive effects on thawing loss and b* (Tables S5 and S6), but the magnitude of the observed effects discard this SNP for future selection purposes.

The myosin is a hexameric protein made of a pair of heavy chains and two pairs of light chains, which interacts with actin and configures the actomyosin complex. During the transformation of muscle to meat, the actomyosin complex participates in proteolysis with enzymatic activities hydrolysing ATP and releasing energy being able to affect processes related to changes on tenderness and water holding capacity on meat (Puolanne & Halonen, 2010). The *MYH3* gene codes for the Myosin Heavy Chain 3 protein, which is a member of the MYH family. In the present study, significant associations on cooking loss of four *MYH3* SNPs mapped on SSC12 were observed (Table 5). These four effects are actually the same due to these SNPs being in strong linkage disequilibrium (Figure 1d). Although a significant association with colour (a* and b*) of the *ss478937995* SNP, which corresponds to the SNP here named as *rs81211437A > G*, has been reported (Liu et al., 2015) in a Chinese Erhualian indigenous pig population, to our knowledge, there are no effects of *MYH3* SNPs on water loss in pigs reported so far. The strong effect on CL advises to take into account this SNP in future works.

In the present study, we observed for the first time, SNPs mapped on *CASP3, CASP9*, *CTSL* and *MYH3* genes affecting different meat quality traits. Moreover, the most relevant results of the association analyses were those observed between *CAPN1_rs81358667G > A* and *CASP3_rs319658214G > T* SNPs on shear force in cooked meat and *PRKAG3_rs319678464G > C* or *PRKAG3_rs330427832C > T, MYH3_rs330483504C > A MYH3_rs81437544T > C, MYH3_rs341130038A > C, or MYH3_rs81211437A > G, CASP3_rs319658214G > T* and *CTSL_rs332171512A > G* or *CTSL_rs321623592A > T* SNPs on different water loss traits.

It is arduous to perform genetic improvement by traditional breeding methods for quality traits based on laborious and expensive determination of phenotypes, especially when phenotype must be recorded more than two years after the birth of the slaughtered animals. Marker assisted selection through the genotyping of several markers should be a cost-effective approach for improving these traits. Similar studies searching for DNA markers for selecting traits of interest such as intramuscular fat and fatty acid profile in this Iberian closed line are being carried out (Muñoz, García-Casco, et al., 2018). Combining all the results obtained in the different studies, causality effects aside, a next step would allow the design of a SNP panel for selecting meat quality traits including the SNPs reported here mapping on *PRKAG3, CAPN1, CASP3, CTSL* and *MYH3* genes. In Iberian pig breeding schemes, production traits such as the yield of premium cuts (hams, shoulders and loins) and average daily gain should not be neglected. The negative correlation between productive and quality traits (García Casco et al., 2014), even between some quality traits (as the observed antagonistic effect of *PRKAG3* SNPs on WHC and colour or the strong positive genetic correlation between DL and CFL with colour a*), makes it more complex to improve all the traits within the same breeding strategy and work is needed to optimize multiple trait selection. The pre-selection of young boars with the most favourable genotype for meat quality traits and similar estimated breeding values for productive traits should be an important step in genetic improvement of these traits. The use of these SNPs in other Iberian populations or varieties, even more in the crossbreeding schemes with Duroc, should be evaluated.

5 | CONCLUSIONS

In the present study, a set of meat quality traits was characterized in purebred Iberian pigs fattened under a traditional free-range production system.

Moderate heritability values for shear force on cooked meat, cooking loss and redness Minolta (a*) values suggest that these trait could be included in a breeding scheme. The strong genetic correlations found between the three WHC traits (TL, CL and CFL), shear force on cooked meat and WHC traits, lightness Minolta (L*) with WHC traits and myoglobin content with a*, support a common genetic background shared by these traits.

Regarding the association analysis between these traits and a customized panel of 32 SNPs, 37 significant associations of SNPs mapped on SSC2 (*CAPN1* and *MYOD* genes), SSC6 (*CAPNS1*, *CAPNS2* and *CASP9* genes), SSC10 (*CTSL* gene), *SCC12* (*MYH3* gene) and SSC15 (*CASP3* and *PRKAG3* gene) on different quality traits were observed.

Two SNPs mapping on *PRKAG3* (*rs319678464G > C* and *rs330427832C > T*), one on *MYH3* (*rs81437544T > C*), one on *CASP3* (*rs319658214G > T*) and two on *CTSL* (*rs332171512A > G* and *rs321623592A > T*) showed the larger effects on water losses. One SNP mapping on *CAPN1* (*rs81358667G > A*) and one in *CASP3 (rs319658214G > T)* showed the highest effects on shear force. The SNPs mapping on *PRKAG3* showed the highest effects on colour. These significant associations are described at first time for the studied meat traits on Iberian pig. Although further studies should be carried out, these SNPs could be considered for their inclusion in Iberian breeding programmes for the pre-selection of young boars.

ACKNOWLEDGEMENTS

This work was financially supported by CON15-078, CON17-125 (INIA-SRC) and IDI-20171141 (CDTI) grants. Miguel Ángel Fernández-Barroso was funded by a FPI Ph.D. grant from the INIA institution. We want to thank to Sánchez Romero Carvajal (SRC) enterprise for the technical support, especially to its manager Jose María Pariente. We also acknowledge Dr. Emiliano de Pedro (University of Córdoba) for the IMF measurements by NIRS method. We are gratefully acknowledged to our colleagues, Yolanda Núñez, Fabián García and Rita Benitez (INIA) for their technical assistance.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

ORCID

Miguel Á. Fernández-Barroso https://orcid.org/0000-0001-5309-9321
María Muñoz https://orcid.org/0000-0002-7018-6977

REFERENCES

Alberti, P., Panea, B., Ripoll, G., Sañudo, C., Olleta, J. L., Hegueruela, I., …Serra, X. (2005). Medición del color. Estandarización de las metodologías para evaluar la calidad del producto (animal vivo, canal, carne y grasa) en los rumiantes. Monografías INIA: Serie Ganadería Nº 3. Págs. 216–225.

Allais, S., Journaux, L., Levéziel, H., Payet-Duprat, N., Raynaud, P., Hocquette, J.-F., … Bernard-Capel, C. (2011). Effects of polymorphisms in the calpastatin and μ-calpain genes on meat tenderness in 3 French beef breeds. *Journal of Animal Science*, *89*, 1–11.

Almeida, J., Bressan, M. C., Santos-Silva, J., Moreira, O., Bettencourt, C., & Gama, L. T. (2018). Physicochemical characteristics and sensory attributes of meat from heavy-weight Iberian and F1 large white × landrace pigs finished intensively or in free-range conditions. *Journal of Animal Science*, *96*(7), 2734–2746.

Alves, E., Ovilo, C., Rodriguez, M. C., & Silio, L. (2003). Mitochondrial DNA sequence variation and phylogenetic relationships among Iberian pigs and other domestic and wild pig populations. *Animal Genetics*, *34*(5), 319–324. https://doi.org/10.1046/j.1365-2052.2003.01010.x

Avilés, C., Juárez, M., Peña, F., Domenech, V., Clemente, I., & Molina, A. (2013). Association of single nucleotide polymorphisms in CAPN1 and CAST genes with beef tenderness from Spanish commercial feedlots. *Czech Journal Animal Science*, *58*, 2013 (10): 479–487.

Baby, S., Hyeong, K. E., Lee, Y. M., Jung, J. H., Oh, D. Y., Nam, K. C., … Kim, J. J. (2014). Evaluation of genome based estimated breeding values for meat quality in a Berkshire population using high density single nucleotide polymorphism chips. *Asian-Australasian Journal of Animal Sciences*, *27*(11), 1540–1547. https://doi.org/10.5713/ajas.2014.14371

Barrett, J. C., Fry, B., Maller, J., & Daly, M. J. (2005). Haploview: Analysis and visualization of LD and haplotype maps. *Bioinformatics*, *21*(2), 263–265. https://doi.org/10.1093/bioinformatics/bth457

Chen, J. F., Dai, L. H., Peng, J., Li, J. L., Zheng, R., Zuo, B., … Jiang, S. W. (2008). New Evidence of Alleles (*V199I and G52S*) at the *PRKAG3 (RN)* Locus Affecting Pork Meat Quality. Asian-Aust. *Journal Animal Science*, *21*(4), 471–477.

Ciobanu, D., Bastiaansen, J., Malek, M., Helm, J., John Woollard, J., Plastow, G., & Rothschild, M. (2001). Evidence for New Alleles in the Protein Kinase Adenosine Monophosphate-Activated ɣ3-Subunit Gene Associated With Low Glycogen Content in Pig Skeletal Muscle and Improved Meat Quality. *Genetics*, *159*, 1151–1162.

Combes, S., Lepetit, J., Darche, B., & Lebas, F. (2004). Effect of cooking temperature and cooking time on Warner-Bratzler tenderness measurement and collagen content in rabbit meat. *Meat Science*, *66*(1), 91–96. https://doi.org/10.1016/S0309-1740(03)00019-6

Davoli, R., Schivazappa, C., Zambonelli, P., Braglia, S., Rossi, A., & Virgili, R. (2017). Association Study between Single Nucleotide Polymorphisms in Porcine Genes and Pork Quality Traits for Fresh Consumption and Processing into Italian Dry-Cured Ham. *Meat Science*, *126*, 73–81. https://doi.org/10.1016/j.meatsci.2016.11.018

De Koning, D. J., Harlizius, B., Rattink, A. P., Groenen, M. A. M., Brascamp, E. W., & Van Arendonk, J. A. M. (2001). Detection and characterization of quantitative trait loci for meat quality traits in pigs. *Journal of Animal Science*, *79*(11), 2812–2819. https://doi.org/10.2527/2001.79112812x

Estévez, M., Morcuende, D., & Cava López, R. (2003). Physicochemical characteristics of M. Longissimus dorsi from three lines of free-range reared Iberian pigs slaughtered at 90 kg live-weight and commercial pigs: A comparative study. *Meat Science*, *64*(4), 499–506. https://doi.org/10.1016/S0309-1740(02)00228-0

Fontanesi, L., & Samorè, A. B. (2016). Genomic selection in pigs: State of the art and perspectives. *Italian Journal of Animal Science 15*(2).

Gandolfi, G., Cinar, M. U., Ponsuksili, S., Wimmers, K., Tesfaye, D., Looft, C., … Davoli, R. (2011). Association of *PPARGC1A* and *CAPNS1* gene polymorphisms and expression with meat quality traits in pigs. *Meat Science*, *89*(4), 478–485. https://doi.org/10.1016/j.meatsci.2011.05.015

Gandolfi, G., Pomponio, L., Ertbjerg, P., Karlsson, A. H., Nanni Costa, L., Lametsch, R., … Davoli, R. (2011). Investigation on *CAST, CAPN1* and *CAPN3* Porcine Gene Polymorphisms and Expression in Relation to Post-Mortem Calpain Activity in Muscle and Meat Quality. *Meat Science*, *88*(4), 694–700. https://doi.org/10.1016/j.meatsci.2011.02.031

García-Casco, J. M., Muñoz, M., Silió, L., & Rodríguez-Valdovinos, C. (2014). Genotype by environment interaction for carcass traits and intramuscular fat content in heavy Iberian pigs fattened in two different free-range systems. *Spanish Journal of Agricultural Research.*, *12*(2), 388–395. https://doi.org/10.5424/sjar/2014122-4840

Gjerlaug-Enger, E., Aass, L., Odegård, J., & Vangen, O. (2010). Genetic parameters of meat quality traits in two pig breeds measured by rapid methods. *Animal*, *4*(11), 1832–1843. https://doi.org/10.1017/S175173111000114X

Goll, D. E., Thompson, V. F., Li, H., Wei, W., & Cong, J. (2003). The Calpain System. *Physiological Review*, *1990*(83), 731–801. https://doi.org/10.1152/physrev.00029.2002

Groeneveld, E., Kovak, M., & Mielenz, N. (2010). *6.* Users' guide and reference manual, Version 6.0. *Institute of farm animal genetics*, Vol. *VCE*. Neustadt, Germany:

Han, X., Jiang, T., Yang, H., Zhang, Q., Wang, W., Fan, B., & Liu, B. (2012). Investigation of four porcine candidate genes (*H-FABP, MYOD1, UCP3 and MASTR*) for meat quality traits in Large White pigs. *Molecular Biology Reports*, *39*, 6599–6605.

Hill, W. G. (2008). Estimation, effectiveness and opportunities of long term genetic improvement in animals and maize. *Lohmann Information.*, *43*(1).

Honikel, K. (1998). Reference methods for the assessment of physical characteristics of meat. *Meat Science*, *49*(4), 447–457. https://doi.org/10.1016/S0309-1740(98)00034-5

Hornsey, H. C. (1956). The colour of cooked cured pork. 1. Estimation of the nitric oxide–haem pigments. *Journal of the Science of Food and Agriculture*, *7*, 534–541.

Ibáñez-Escriche, N., Magallón, E., Gonzalez, E., Tejeda, J. F., & Noguera, J. L. (2016). Genetic parameters and crossbreeding effects of fat deposition and fatty acid profiles in Iberian pig lines. *Journal of Animal Science*, *94*, 28–37. https://doi.org/10.2527/jas.2015-9433

Juárez, M., Clemente, I., Polvillo, O., & Molina, A. (2009). Meat quality of tenderloin from Iberian pigs as affected by breed strain and crossbreeding. *Meat Science*, *81*, 573–579. https://doi.org/10.1016/j.meatsci.2008.10.016

Jung, J. H., Kim, C. W., Park, B. Y., Choi, J. S., & Park, H. C. (2011). Genetic parameter estimates for meat quality traits in Berkshire pigs. *Journal of Animal Science Technology*, *53*, 289–296.

Kemp, C. M., Sensky, P. L., Bardsley, R. G., Buttery, P. J., & Parr, T. (2010). Tenderness - An enzymatic view. *Meat Science*, *84*(2), 248–256. https://doi.org/10.1016/j.meatsci.2009.06.008

Kim, J. M., Choi, B. D., Kim, B. C., Park, S. S., & Hong, K. C. (2009). Associations of the variation in the porcine myogenin gene with muscle fibre characteristics, lean meat production and meat quality traits. *Journal of Animal Breeding and Genetics*, *126*(2), 134–141. https://doi.org/10.1111/j.1439-0388.2008.00724.x

Koohmaraie, M., & Geesink, G. H. (2006). Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. *Meat Science*, *74*, 34–43. https://doi.org/10.1016/j.meatsci.2006.04.025

Larzul, C., Lefaucheur, L., Ecolan, P., Gogué, J., Talmant, A., Sellier, P., … Monin, G. (1997). Phenotypic and genetic parameters for longissimus muscle fiber characteristics in relation to growth, carcass, and meat quality traits in large white pigs. *Journal of Animal Science*, *75*(12), 3126–3137. https://doi.org/10.2527/1997.75123126x

Lee, E. A., Kim, J. M., Lim, K. S., Ryu, Y. C., Jeon, W. M., & Hong, K. C. (2012). Effects of variation in porcine *MYOD1* gene on muscle fiber characteristics, lean meat production and meat quality traits. *Meat Science*, *92*, 36–43. https://doi.org/10.1016/j.meatsci.2012.03.018

Lee, J. H., Song, K. D., Lee, H. K., Cho, K. H., Park, H. C., & Park, K. D. (2015). Genetic Parameters of Reproductive and Meat Quality Traits in Korean Berkshire Pigs. Asian Australas. *Journal Animal Science*, *28*(10), 1388–1393.

Listrat, A., Lebret, B., Louveau, I., Astruc, T., Bonnet, M., Lefaucheur, L., … Bugeon, J. (2016). How muscle structure and composition influence meat and flesh quality. *Scientific World Journal*, *2016*, https://doi.org/10.1155/2016/3182746

Liu, X., Xiong, X., Yang, J., Zhou, L., Yang, B., Ai, H., & Huang, L. (2015). Genome-wide association analyses for meat quality traits in Chinese Erhualian pigs and a Western Duroc x (Landrace × Yorkshire) commercial population. *Genetics Selection Evolution*, *47*(1), 1–11. https://doi.org/10.1186/s12711-015-0120-x

López-Bote, C. J. (1998). Sustained utilization of the Iberian pig breed. *Meat Science.*, *49*, 17–27. https://doi.org/10.1016/S0309-1740(98)00072-2

Mayoral, A. I., Dorado, M., Guillen, M. T., Robina, A., Vivo, J. M., Vázquez, C., & Ruiz, J. (1999). Development of meat and carcass quality characteristics in Iberian pigs reared outdoors. *Meat Science*, *52*, 315–324. https://doi.org/10.1016/S0309-1740(99)00008-X

Miar, Y., Plastow, G. S., Moore, S. S., Manafiazar, G., Charagu, P., Kemp, R. A., & Wang, Z. (2014). Genetic and phenotypic parameters for carcass and meat quality traits in commercial crossbred pigs. *Journal of Animal Science*, *92*(7), 2869–2884. https://doi.org/10.2527/jas.2014-7685

Milan, D., Jeon, J. T., Looft, C., Amarger, V., Robic, A., Thelander, M., … Andersson, L. (2000). A mutation in *PRKAG3* associated with excess glycogen content in pig skeletal muscle. *Science*, *288*, 1248–1251. https://doi.org/10.1126/science.288.5469.1248

Morales, R., Guerrero, L., Serra, X., & Gou, P. (2007). Instrumental evaluation of defective texture in dry-cured hams. *Meat Science*, *76*(3), 536–542. https://doi.org/10.1016/j.meatsci.2007.01.009

Moskvina, V., & Schmidt, K. M. (2008). On multiple-testing correction in genome-wide association studies. *Genetic Epidemiology*, *32*(6), 567–573. https://doi.org/10.1002/gepi.20331

Muñoz, M., Bozzi, R., García, F., Núñez, Y., Geraci, C., Crovetti, A., … Óvilo, C. (2018). Diversity across major and candidate genes in European local pig breeds. *PLoS One*, *13*(11), e0207475. https://doi.org/10.1371/journal.pone.0207475

Muñoz, M., García-Casco, J. M., Alves, E., Benítez, R., Barragán, C., Caraballo, C., … Silió, L. (2020). Development of a 64 SNV panel for breed authentication in Iberian pigs and their derived meat products. *Meat Science*, *167*, 108152. https://doi.org/10.1016/j.meatsci.2020.108152

Muñoz, M., García-Casco, J. M., Caraballo, C., Fernández-Barroso, M. A., Sánchez-Esquiliche, F., Gómez-Carballar, F., … Silió, L. (2018). Identification of candidate genes and regulatory factors underlying intramuscular fat content through *Longissimus dorsi* transcriptome analyses in heavy Iberian pigs. *Frontiers in Genetics*, *682*(9), 1–16. https://doi.org/10.3389/fgene.2018.00608

Muñoz, M., Sánchez-Esquiliche, F., Caraballo, C., Gómez, F., Pariente, J. M., Silió, L., …García-Casco, J. M. (2018). Animal breeding scheme applied to the quality of pure Iberian montanera pigs. *Archivos Zootecnia Proceedings* IX Simposio Internacional sobre el. Cerdo Mediterráneo.

Newcom, D. W., Stalder, K. J., Baas, T. J., Goodwin, R. N., Parrish, F. C., & Wiegand, B. R. (2004). Breed differences and genetic parameters of myoglobin concentration in porcine longissimus muscle. *Journal of Animal Science*, *82*(8), 2264–2268.

Nieto, R., García-Casco, J., Lara, L., Palma-Granados, P., Izquierdo, M., Hernández, F., …Batorek-Lukac, N. (2019). European local pig breeds-diversity and performance. A study of project TREASURE. Chapter 9, Iberian, pig. 115–140.

Nonneman, D. J., Shackelford, S. D., King, D. A., Wheeler, T. L., Wiedmann, R. T., Snelling, W. M., & Rohrer, G. A. (2013). Genome-wide association of meat quality traits and tenderness in swine. *Journal Animal Science*, *91*, 4043–4050.

Nyholt, D. R. (2004). A simple correction for multiple testing for SNPs in linkage disequilibrium with each other. *American Journal of Human Genetics*, *74*, 765–769.

Ollivier, L. (2009). European pig genetic diversity: A minireview. *Animal*, *3*(07), 915–924. https://doi.org/10.1017/s1751731109004297

Otto, G., Roehe, R., Looft, H., Thoelking, L., Knap, P. W., Rothschild, M. F., … Kalma, E. (2007). Associations of DNA markers with meat quality traits in pigs with emphasis on drip loss. *Meat Science*, *75*(2), 185–195. https://doi.org/10.1016/j.meatsci.2006.03.022

Ouali, A., Herrera-Mendez, C. H., Coulis, G., Becila, S., Boudjellal, A., Aubry, L., & Sentandreu, M. A. (2006). Revisiting the conversion of muscle into meat and the underlying mechanisms. *Meat Science*, *74*, 44–58. https://doi.org/10.1016/j.meatsci.2006.05.010

Page, B. T., Casas, E., Heaton, M. P., Cullen, N. G., Hyndman, D. L., & Morris, C. A. (2002). Evaluation of single-nucleotide polymorphisms in *CAPN1* for association with meat tenderness in cattle. *Journal of Animal Science*, *80*, 3077–3085.

Pateiro, M., Franco, D., Carril, J. A., & Lorenzo, J. M. (2015). Changes on physico-chemical properties, lipid oxidation and volatile compounds during the manufacture of Celta dry-cured loin. *Journal of Food Science and Technology*, *52*(8), 4808–4818. https://doi.org/10.1007/s13197-014-1561-x

Pérez-Enciso, M., & Misztal, I. (2004). Qxpak: A versatile mixed model application for genetical genomics and QTL analyses. *Bioinformatics*, *20*(16), 2792–2798. https://doi.org/10.1093/bioinformatics/bth331

Ponsuksili, S., Jonas, E., Murani, E., Phatsara, C., Srikanchai, T., Walz, C. et al (2008). Trait correlated expression combined with expression QTL analysis reveals pathways and candidate genes affecting water holding capacity of muscle. *BMC Biological Genomics*, *9*, 367.

Pugliese, C., Bozzi, R., Campodoni, G., Acciaioli, A., Franci, O., & Gandini, G. (2005). Performance of Cinta Senese pigs reared outdoors and indoors. 1. Meat and subcutaneous fat characteristics. *Meat Science*, *69*, 459–464. https://doi.org/10.1016/j.meatsci.2004.09.001

Puolanne, E., & Halonen, M. (2010). Theoretical aspects of water-holding in meat. *Meat Science*, *86*(1), 151–165. https://doi.org/10.1016/j.meatsci.2010.04.038

Ramírez, M. R., & Cava, R. (2007). Effect of Iberian × Duroc genotype on dry-cured loin quality. *Meat Science*, *76*(2), 333–341. https://doi.org/10.1016/j.meatsci.2006.11.017

Rey, A. I., Daza, A., López-Carrasco, C., & López-Bote, C. J. (2006). Quantitative study of the α- and γ-tocopherols accumulation in muscle and backfat from Iberian pigs kept free-range as affected by time of free-range feeding or weight gain. *Animal Science*, *82*(06), 901. https://doi.org/10.1017/asc2006113

Rohrer, G. A., Nonneman, D. J., Miller, R. K., Zerby, H., & Moeller, S. J. (2012). Association of single nucleotide polymorphism (SNP) markers in candidate genes and QTL regions with pork quality traits in commercial pigs. *Meat Science*, *92*(4), 511–518. https://doi.org/10.1016/j.meatsci.2012.05.020

SanCristobal, M., Chevalet, C., Haley, C. S., Joosten, R., Rattink, A. P., Harlizius, B., … Cardellino, R. (2006). Genetic diversity within and between European pig breeds using microsatellite markers. *Animal Genetics*, *37*(3), 189–198. https://doi.org/10.1111/j.1365-2052.2005.01385.x

Sentandreu, M. A., Coulis, G., & Ouali, A. (2002). Role of muscle endopeptidases and their inhibitors in meat tenderness. *Trends in Food Science and Technology*, *13*, 400–421. https://doi.org/10.1016/S0924-2244(02)00188-7

Serra, X., Gil, F., Pérez-Enciso, M., Oliver, M. A., Vázquez, J. M., Gispert, M., & Noguera, J. L. (1998). A comparison of carcass, meat quality and histochemical characteristics of Iberian (Guadyerbas line) and Landrace pigs. *Livestock Production Science*, *56*(3), 215–223. https://doi.org/10.1016/S0301-6226(98)00151-1

Silió, L. (2000). Iberian pig breeding program. In S. Galal, J. Boyazoglou, & K. Hammond (Eds.), *Developing Breeding Strategies for Lower Input* (pp. 511–519). Environments, ICAR, Rome: Animal Production.

Škrlep, M., Čandek-Potokar, M., Žlender, B., Robert, N., Santé-Lhoutellier, V., & Gou, P. (2012). PRKAG3 and CAST genetic polymorphisms and quality traits of dry-cured hams—III. Associations in Slovenian dry-cured ham Kraški pršut and their dependence on processing. *Meat Science*, *92*(4), 360–365. https://doi.org/10.1016/j.meatsci.2012.06.021

Škrlep, M., Kavar, T., & Čandek-Potokar, M. (2010). Comparison of *PRKAG3* and *RYR1* gene effect on carcass traits and meat quality in Slovenian commercial pigs. *Czech Journal Animal Science*, *55*(4), 149–159.

Solís, M., de Pedro, E., Garrido, A., & García-Olmo, J. (2001). Evaluación de la composición del lomo de cerdo ibérico mediante la tecnología NIRS. *ITEA*, *22*, 613–615.

Sorimachi, H., Hata, S., & Yasuko, O. (2011). Impact of genetic insights into calpain biology. *Journal Biochemistry*, *150*(1), 23–37. https://doi.org/10.1093/jb/mvr070

Storey, J. D., Bass, A. J., Dabney, A., & Robinson, D. (2019). Qvalue: Q-value estimation for false discovery rate control. R package version 2.16.0, http://github.com/jdstorey/qvalue

Sun, X., Wu, X., Fan, Y., Mao, Y., Ji, D., Huang, B., & Yang, Z. (2018). Effects of polymorphisms in *CAPN1* and *CAST* genes on meat tenderness of Chinese Simmental cattle. *Archives Animal Breeding*, *61*, 433–439.

Suzuki, K., Irie, M., Kadowaki, H., Shibata, T., Kumagai, M., & Nishida, A. (2005). Genetic parameter estimates of meat quality traits in Duroc pigs selected for average daily gain, longissimus muscle area, backfat thickness, and intramuscular fat content. *Journal of Animal Science*, *83*(9), 2058–2065. https://doi.org/10.2527/2005.8392058x

te Pas, M. F., Soumillion, A., Harders, F. L., Verburg, F. J., van den Bosch, T. J., Galesloot, P., & Meuwissen, T. H. (1999). Influences of myogenin genotypes on birth weight, growth rate, carcass weight, backfat thickness, and lean weight of pigs. *Journal of Animal Science*, *77*(9), 2352–2356.

Team, R. (2015). *RStudio: Integrated development for R*. Boston, MA: RStudio, Inc., Retrieved from http://www.rstudio.com

Tejerina, D., García-Torres, S., & Cava, R. (2012). Water-Holding capacity and instrumental texture properties of M. Longissimus Dorsi and M. Serratus Ventralis from Iberian Pigs as affected by the production system. *Livestock Science*, *148*(1–2), 46–51.

Thornton, C., Snowden, M. A., & Carling, D. (1998). Identification of a novel amp-activated protein kinase beta subunit isoform that is highly expressed in skeletal muscle. *Journal of Biological Chemistry*, *273*, 12443–12450.

Toro, M. A., Rodrigáñez, J., Silió, L., & Rodríguez, C. (2000). Genealogical analysis of a closed herd of black hairless Iberian

pigs. *Conservation Biology*, *14*, 1843–1851. https://doi.org/10.1111/j.1523-1739.2000.99322.x

Van Wijk, H. J., Arts, D. J., Matthews, J. O., Webster, M., Ducro, B. J., & Knol, E. F. (2005). Genetic parameters for carcass composition and pork quality estimated in a commercial production chain. *Journal of Animal Science*, *83*, 324–333.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., van Roy, N., Paepe, D. E., & Speleman, F. (2002). Accurate normalization of Real-Time Quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*(711), 34–41.

Ventanas, S., Ventanas, J., Jurado, A., & Estevez, M. (2006). Quality traits in muscle biceps femoris and back-fat from purebred Iberian and reciprocal Iberian x Duroc crossbred pigs. *Meat Science*, *73*, 651–659.

Ventanas, S., Ventanas, J., Ruiz, J., & Estevez, M. (2005). Iberian pigs for the development of high-quality cured products. *Recent Res Develop Agricultural & Food Chem*. 6(27–53). Research Signpost, Kerala, India.

Weiler, U., Appell, H. J., Kremser, M., Hofacker, S., & Claus, R. (1995). Consequences of selection on muscle composition. A comparative study on gracilis muscle in wild and domestic pigs. *Journal of Veterinary Medicine, Series C*, *24*, 77–80.

Whipple, G., Koohmaraie, M., Dikeman, M. E., Crouse, J. D., Hunt, M. C., & Klemm, R. D. (1990). Evaluation of attributes that affect longissimus muscle tenderness in Bos taurus and Bos indicus cattle. *Journal of Animal Science*, *68*, 2716–2728. https://doi.org/10.2527/1990.6892716x

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Fernández-Barroso MÁ, Silió L, Rodríguez C, et al. Genetic parameter estimation and gene association analyses for meat quality traits in open-air free-range Iberian pigs. *J Anim Breed Genet*. 2020;137:581–598. https://doi.org/10.1111/jbg.12498

CAPÍTULO II

Use of NIRS for the assessment of meat quality traits in open-air free-range Iberian pigs.

Fernández-Barroso M.A., Parrini S., Muñoz M., Palma-Granados P., Matos G., Ramírez L., Crovetti A., Juan María García-Casco J.M., Bozzi R. 2021. Journal of food composition and analysis. 102, 104018. https://doi.org/10.1016/j.jfca.2021.104018.

Journal of Food Composition and Analysis 102 (2021) 104018

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original Research Article

Use of NIRS for the assessment of meat quality traits in open-air free-range Iberian pigs

Miguel Ángel Fernández-Barroso [a,b,*], Silvia Parrini [c], María Muñoz [a,b], Patricia Palma-Granados [a,b], Gema Matos [d], Luisa Ramírez [d], Alessandro Crovetti [c], Juan María García-Casco [a,b], Riccardo Bozzi [c]

[a] Centro Nacional de I+D del Cerdo Ibérico, INIA, Zafra, 06300, Spain
[b] INIA, Departamento de Mejora Genética Animal, Madrid, 28040, Spain
[c] Dipartimento di Scienze e Tecnologie Agrarie, Alimentari, Ambientali e Forestali (DAGRI), Università di Firenze, Italy
[d] Sánchez Romero Carvajal—Jabugo, SRC, Huelva, 21290, Spain

ARTICLE INFO

Keywords:
NIRS
Iberian pig
Meat quality
Intact and minced samples

ABSTRACT

Near infrared spectroscopy (NIRS) can be useful in order to determine meat quality traits as a rapid and non-destructive technique. The aim of the present study is to assess the accuracy of NIRS technique to determine meat quality traits on *Longissimus thoracis et lumborum* muscle of open-air free-range Iberian pig (n = 287) both in intact and minced samples. Traits were measured by instrumental-chemical techniques: colour (L*, a* and b*), myoglobin content, centrifuge force water loss and texture: shear force and texture profile analysis (TPA: hardness, cohesiveness, springiness, chewiness). Calibration models between instrumental-chemical measures and NIRS spectral data were developed employing partial least square regressions (PLS). The samples were split in two random datasets (80 % in training set, 20 % in external validation set). An internal full cross-validation method was applied. Results were evaluated in terms of coefficient of determination (R^2), root-mean-square error (RMSE), residual prediction deviation (RPD) and range error ratio (RER). Full spectral range was used to develop mathematical equations. As regard external validation procedure, the highest coefficients of determination (R^2p) in intact loin samples were achieved for hardness, redness (a*) and yellowness (b*) ($0.7<R^2p<0.8$), while for minced samples lightness (L*), myoglobin content and texture parameters obtained always $R^2p>0.7$. The models developed suggest the ability of NIRS for quantitative prediction of shear force and for a rough classification of colour parameters L*, a*, b* in minced samples as well as hardness in intact samples.

1. Introduction

The traditional Iberian pig production system is characterized by an open-air, free-range fattening period named *Montanera* based on *ad libitum* intake of grass and acorns developed in the *Dehesa* ecosystem (López-Bote, 1998). The high organoleptic meat quality is influenced by the interaction between this rearing system and the genetic character- istics of the breed. Iberian pigs have a slower growth rate, higher po- tential of fat deposition and higher proportion of monounsaturated fatty acids with respect to other breeds.

Therefore, high meat quality, which is increasingly demanded by consumers, is one of the main aims of the pork processing industry. Usually, meat characteristics are determined by classical chemical, technological and sensorial analyses. The implementation of these techniques is often expensive, invasive and time consuming; consequently, those methodologies are not adapted for real-time analysis and sometimes generate hazardous waste (Tejerina et al., 2018). This is particularly relevant when meat quality traits are included in breeding programs since a large amount of data are needed, requiring cheaper and less laborious techniques.

Near infrared spectroscopy (NIRS), based on the absorption of electromagnetic radiation at wavelengths in the 780–2500 nm range, is a recognized methodology for some chemical analyses both in food and feed. Near infrared spectra of foods include broad bands arising from overlapping absorption profiles corresponding mainly to overtones and combinations of vibrational modes involving C–H, OH and NH—chemical bonds (Osborne, 2000). Nevertheless, this technique requires calibration against a reference method for each parameter of interest

* Corresponding author at: Centro de I+D en Cerdo Ibérico, INIA, Zafra, Spain.
E-mail address: fernandez.miguel.inia@gmail.com (M.Á. Fernández-Barroso).

https://doi.org/10.1016/j.jfca.2021.104018
Received 8 October 2020; Received in revised form 15 May 2021; Accepted 1 June 2021
Available online 4 June 2021

and it requires a multivariate analysis able to associate the spectral absorption of the near infrared region with laboratory data. A large database with high levels of variability representing all possible characteristics of the samples is essential to the development of robust and accurate NIRS predictions (Parrini et al., 2017). NIRS could be useful to replace traditional methods of analysis. In some studies, it has been applied for rapid and non-destructive determination of meat quality traits. Prieto et al. (2009) reported that the NIRS technique has a high potential to predict chemical content of meat and to categorise samples based on their meat quality characteristics. Also, Monteiro-Balage et al. (2015) described the potential of NIRS to correctly classify pork samples into two categories (tough and tender), when they used the visible spectrum combined with a part of the near infrared spectrum. In addition, Prieto et al. (2015) showed the capacity of NIRS to discriminate between pork quality of Lacombe, Duroc and Iberian loins applying the technology directly in processing plants. Nevertheless, apart from its discriminatory ability, the same authors (Monteiro-Balage et al., 2015; Prieto et al., 2009) reported low NIRS performance to accurately estimate quality traits, due to different factors including the type of instrument and spectra regions, the statistical methods adopted, the precision of reference methods as well as the number and type of meat samples that, in the case of fresh meat, are linked to the heterogeneity of intact samples and high water content. The use of Fourier Transform in NIRS (FT-NIRS), which includes improvements in signal-to-noise ratio, spectral resolution and wavelength accuracy, could be a key factor to allow qualitative analyses of the traits, improving measurement precision, time of analysis, and costs.

Several studies have used NIRS as a rapid method applicable to Iberian pig products (Cáceres-Nevado et al., 2019; Pérez-Marín et al., 2009; Solís et al., 2001; Tejerina et al., 2018; Zamora-Rojas et al., 2012, 2013). Overall, these NIRS studies were focused on determining chemical composition, intramuscular fat content and fatty acid profiles, and just few compared intact and minced samples (Cáceres-Nevado et al., 2019; Ortiz et al., 2020; Pérez-Marín et al., 2009). The possibility of determining meat quality traits directly on the intact piece could allow both to avoid meat destruction and to reduce the time required for the analysis. Therefore, it is important to investigate these aspects further.

The aim of the present study was to assess the accuracy of the NIRS technique to determine several meat quality traits in intact and minced samples of loins belonging to Iberian pigs fattened in an open-air free-range system using the full near infrared region. As far as we know, this is the first time that the feasibility of NIRS technique is evaluated in this porcine breed and production system, as a quick and clean technique to simultaneously measure this set of meat quality traits in just one run, which includes water holding capacity, myoglobin content, instrumental colour and texture parameters.

Due to the genetic uniqueness of Iberian pig, associated with lack of introgression of Asian and other lean European breeds alleles (Alves et al., 2003; Ollivier, 2009; SanCristobal et al., 2006), the results found in studies with other pig breeds may not be extrapolated to the Iberian. This simultaneous measurement would be very important for the Iberian pig meat industry and particularly in breeding programs including meat quality parameters as a selection goal, since a large number of records are required.

2. Materials and methods

2.1. Animal material

Pigs included in the current study were castrated males belonging to a commercial population of an Iberian purebred herd that has been closed for approximately 15 years, as described in Fernández-Barroso et al. (2020). The animals were fed under a restricted feeding regime up to approximately 100 kg of body weight and managed in semi-extensive system. Then, they were fattened in an open-air free-range system based on *ad libitum* intake of acorns and grass. A total of 287 animals were slaughtered in the same commercial slaughterhouse in four different slaughter batches in the same year, at an approximate age of 17 months and at an average slaughter weight of 173 kg (SD = 13.7). Animal handling was carried out according to the regulations of the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63/EU about the protection of animals used in research. Research protocols were assessed and approved by the INIA Committee of Ethics in Animal Research, which is the named Institutional Animal Care and Use Committee (IACUC) for the INIA.

2.2. Sample acquisition, texture, colour parameters and chemical determinations

Samples of *Longissimus thoracis et lumborum* (LTL) muscle from 287 pigs were removed from the carcass and both intermuscular fat and connective tissue were separated. The central LTL section was subdivided in two parts (Fig. 1). One part was used for myoglobin content (MB) and centrifuge force loss (CFL) determinations, and the other part was used for determination of texture: shear force, texture profile analysis (TPA: hardness, cohesiveness, springiness, chewiness), instrumental colour parameters (lightness L*, redness a*, and yellowness b*), and for NIRS determination. A quality control method removing the outliers was carried out.

All samples were refrigerated and vacuum-packed individually in nylon/polyethylene bags. After packing, the meat portions corresponding to MB and CFL were introduced in liquid N_2 for approximately 20 s before being stored at −20 °C to quickly freeze them to reduce potential water loss and prevent changes in the chemical state of myoglobin. The remaining samples were stored directly at −20 °C. Prior the analyses, samples were defrosted and tempered until determinations (3–5 °C for 24 h). Myoglobin content, CFL, colour parameters and shear force determinations has been previously described in detail by Fernandez-Barroso et al. (2020).

Myoglobin content was determined following Hornsey (1956) and Alberti et al. (2005) with modifications. Absorbance was converted to mg myoglobin/g muscle following the next equation:

$$\frac{\boldsymbol{Vf} * \boldsymbol{PM} * 1\boldsymbol{kg}}{\boldsymbol{\varepsilon} * \boldsymbol{Psample} * 1000} * \boldsymbol{DO}$$

Where ***Vf*** is the final volume of determination (12.5 ml), **PM** is molecular weight of myoglobin, ***ε*** is a coefficient applied (9.52), ***Psample*** is sample weight (2.5 g), ***DO*** is the optical density absorbance. The method described by Tejerina et al. (2012) was used to measure CFL. Measures of MB and CFL were done in duplicate and then averaged.

A steak of 2 cm thick was cut from the LTL portion for determination of the colour parameters L*, a*, and b* using a colorimeter (CR-400, Konica Minolta, New Jersey, USA) with 8 mm measuring cell aperture, CIE standard illuminant D65, and 2° observer. The analysis was performed at a room temperature of approximately 20−21 °C. Each colour measurement was carried out in triplicate and then averaged.

Texture analyses were performed using a texture analyser (Z2.5 apparatus, Zwick Roell, Ulm, Germany). Shear force was measured by Warner-Bratzler test (Honikel, 1998). Eight 2.5 cm diameter cylinders per sample were prepared. Each cylinder was cut perpendicular to the muscle fibre direction with a Warner-Bratzler blade (HDP/BSW) at a crosshead speed of 1 mm/s and the eight measures were averaged (kg cm^2). Regarding the texture profile analysis (TPA), a total of eight cubes of 1 cm^3 per sample were prepared. Each cube was compressed twice to 50 % of their original height with a 10 cm diameter compression plate (time = 0 s between the two compression cycles), at a crosshead speed of 1 mm/s and perpendicularly to the muscle fibre direction. Force-time curves were recorded and the subsequent rheological parameters were calculated (Bourne, 1978): hardness, cohesiveness, springiness and chewiness. Hardness (N) is defined as the maximum peak force during the first compression cycle, cohesiveness as the ratio between the area of

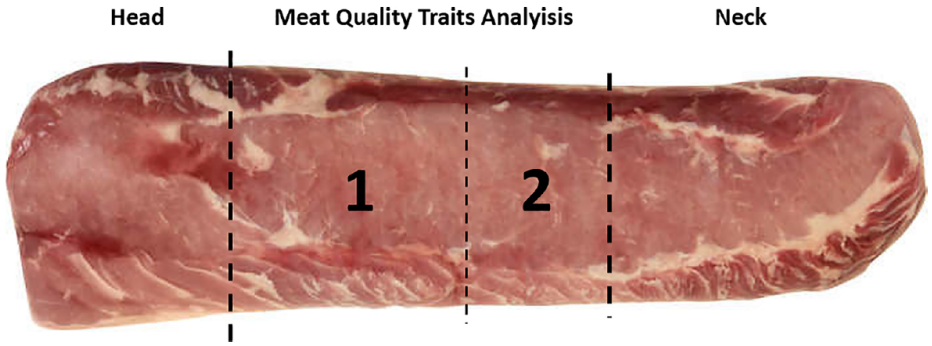


Fig. 1. Assignment of *Longissimus thoracis et lumborum* muscle for meat quality traits. 1: Texture analysis (Warner-Bratzler shear force (SFF) and texture profile analysis (TPA)), instrumental colour parameters (lightness L*, redness a*, and yellowness b*) and NIRS. 2: myoglobin content (MB) and centrifuge force loss (CFL). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the positive force during the second and the first compression cycle, springiness (mm) as the height to which the sample recovers during the time elapsed between the end of the first and the start of the second compression, and chewiness (N x mm) as the work needed to chew a solid sample to a steady state of swallowing (hardness x cohesiveness x springiness).

2.3. FT-NIRS data acquisition

The same samples of approximately 2 cm thick (around 20 g) used for colour parameter measurements were scanned with a FT-NIRS device (Antaris™ II, Thermo Fisher Scientific, Waltham, Massachusetts, USA) at a lab room temperature of approximately 20−21 °C. Spectral data were collected in reflectance mode and recorded as absorbance (log1/R, where R is the reflectance). Two-sample presentation modes were evaluated: intact and minced. Two spectra per sample, one for each side, were scanned for the intact mode using a cylindrical cup spinner with a quartz window. For the minced mode, samples were chopped in a homogeniser and two spectra were scanned for each sample. Each spectral measurement was obtained from 32 scans performed at a wave number resolution of 4 cm^{-1} over the range of 9999 to 4000 cm^{-1} (1000–2500 nm) and corrected against the background spectrum of room environment. Average values of the two different replications for each sample were obtained through the OMNIC™ Software (Thermo Fisher Scientific, Waltham, Massachusetts, USA) both for intact and minced samples.

2.4. FT-NIRS data pre-treatment

NIRS pre-processed spectral data were combined with the results of chemical and technological analysis: MB, CFL, instrumental colour (L*, a*, b*), shear force and TPA (hardness, cohesiveness, springiness and chewiness).

For each trait, an individual calibration model was developed. To optimize the accuracy of the calibration, several mathematical pre-treatments such as multiplicative scatter correction (MSC) and standard normal variate (SNV) were tested. MSC was used in order to remove optical interference and physical effects like particle size and surface blaze (Kapper et al., 2012a). SNV was tested as alternative to MSC in order to remove the multiplicative interferences of scatter and particle size (Barnes et al., 1989). Finally, SNV pre-treatment was not used in the models because the results obtained were worse than MSC pre-treatment.

To optimize the extraction of information from the spectra data, first or second derivatives were used to remove baseline offset variations (Savenije et al., 2006). Also, a Savitzky Golay filter was employed to improve the appearance of peaks obscured by random noise. Finally, outliers' spectra were identified and removed when necessary. Evaluation of outliers and the mathematical pre-treatments were made using TQ Analyst 8.6.12 software (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.5. FT-NIRS calibration and chemometric analysis

The total number of samples was split in two random data sets: a training set (calibration) comprising 80 % of the samples, and a validation set including the remaining 20 % of the samples. An internal full cross-validation using the leave one-out method was applied on the training set. Using the leave one-out method, the number of folds (the data subset portion) is equal to the number of instances in the full data set (Sammut and Webb, 2011).

Partial least squares (PLS) regression was applied to all models using TQ Analyst 8.6.12. For each parameter, the optimum number of factors used by PLS (FPLS) in the model development was the one that determined the lowest error in cross validation and thus avoiding overfitting (Prieto et al., 2014).

The best model for each trait was evaluated in terms of the highest coefficient of determination (R^2) and the lowest root-mean-square error (RMSE), in calibration (R^2c; RMSEC), in cross-validation (R^2cv; RMSECV) and in external validation (R^2p; RMSEP), respectively. RMSE determines the performance of model regression and expresses the difference between the predicted and the measured references values (Lobos et al., 2013). Residual prediction deviation (RPD) was used in order to evaluate goodness of fit and model accuracy; it was calculated as the ratio between the standard deviation of the dataset divided by the root-mean-square error in calibration (RPDc = SD/RMSEC), in cross-validation (RPDcv = SD/RMSECV) and in external validation (RPDp = SD/RMSEP). The model performance can be considered sufficient for a rough screening if RPD is between 1.5 and 2.5 (Williams, 2014). Williams and Sobering (1993) suggested an 'accurate estimation capacity' if RPD values were higher than the limit of 2.5, even though in the following years the limit for the accuracy evaluation was increased to 3 (Williams, 2014). Nevertheless, when reference data variance is low, the values for the R^2 and the RPD cannot be very high (Pérez-Marín et al., 2004). The range error rate (RER) was calculated as the ratio between the range of sample values (reference techniques) and the RMSEP (Max-Min/RMSEP) (Pérez-Marín et al., 2004). RER values in the range of 4–8 suggest the possibility of discriminating between high and low values, while RER values in the range of 8–12 represent the possibility of predicting quantitative data (Barbin et al., 2015; Millmier et al., 2000).

3. Results and discussion

3.1. Descriptive statistics of meat quality traits

A summary of descriptive statistics of the analysed meat quality traits is shown in Table 1. Lightness (L*) was the only parameter that showed certain homogeneity. For the other three traits (yellowness b*, hardness and chewiness), the coefficient of variation was high (from 28.7%–37.0%); and for the remaining traits the coefficient of variation was moderate (between 13.5 % and 18.0 %).

The phenotypic data presented here showed similar values for CFL, L*, a* and MB but slightly higher for b* when compared to animals from the same Iberian pig population presented earlier by Fernández-Barroso et al. (2020). These differences observed within the same population could be due to a strong environmental factor in the open-air free-range production system, where production traits of pigs reared in different seasons are strongly influenced by the weather, the harvest of acorns and the abundance of pastures. Moreover, some of the traits such as water content or fat deposition in muscle are inherently very heterogeneous in pork.

The comparison between the means and dispersion values of the meat quality traits analysed (Table 1) and those reported by other studies on the same traits in Iberian pigs (Estévez et al., 2003; Lopez-bote et al., 2008; Mayoral et al., 1999; Tejerina et al., 2012) is not straightforward due to the different experimental designs in terms of production system, slaughter age, muscle type or instrumental procedures. In addition, in our study, fresh meat samples were frozen, stored and then thawed for subsequent analyses (reference methods and NIRS) instead of performing the analysis on the same day that they were collected. The different freeze/thaw procedures probably affect the values obtained for the traits analysed. However, to carry out these analyses the same day that the samples were collected is unfeasible since an approximate number of 70 samples were collected for each slaughter date in a commercial slaughterhouse.

3.2. NIRS spectra characteristics

Average values of near infrared spectra, both for minced and intact samples from LTL muscle of Iberian pigs are shown in Fig. 2. The spectral information extracted from each type of sample showed a similar pattern, but intact meat seems to absorb slightly more energy than minced samples.

The differences in absorbance can be explained by the texture structure when meat is minced, which interferes with light absorbance, as suggested by Cozzolino et al. (2000) and Fan et al. (2018). Although several studies carried out with the NIRS methodology in other pig breeds focused on evaluating some of the meat quality traits, such as instrumental colour and water holding capacity (Čandek-Potokar et al., 2006; Kapper et al., 2012b; Savenije et al., 2006; Wang et al., 2018), few references reported on the comparison between intact and minced samples. Cáceres-Nevado et al. (2019) also observed slightly higher absorbance of intact samples than minced ones when they analysed moisture, protein and fat content in Iberian pig loins. Ortiz-Somovilla et al. (2006) noted visual differences between different spectra types of pork products (minced and homogenized). Also, it seems that the homogenization of the products results in lower absorption due to the modification of the meat structure.

Furthermore, according to Cozzolino and Murray (2002), near infrared spectra in meat samples present high peaks of absorption (Fig. 2) that are linked to fat (1190 nm or 8400 cm^{-1}), protein (1550 nm or 6451 cm^{-1}) and water (1900 nm or 5260 cm^{-1}).

Table 1
Descriptive statistics for traits from *Longissimus thoracis et lumborum* muscle of Iberian pigs fattened in an open-air free-range system.

Trait	Sample size	Mean	SD	CV (%)	Max	Min
L*	287	43.60	2.93	6.73	53.58	35.90
a*	287	11.48	2.06	17.99	16.29	5.92
b*	287	3.37	1.22	36.22	7.14	1.03
Myoglobin (mg g^{-1} muscle)	287	1.66	0.26	15.66	2.64	1.04
CFL (%)	287	29.98	4.04	13.47	37.49	16.52
Shear Force (kg cm^2)	287	4.39	0.73	16.68	6.61	1.96
Hardness (N)	287	9.34	2.68	28.69	20.31	2.68
Cohesiveness	287	0.28	0.04	14.29	0.39	0.17
Springiness (mm)	287	1.55	0.21	13.55	2.15	0.52
Chewiness (N x mm)	287	4.19	1.55	36.99	8.83	1.20

Sample size: number of samples; SD: standard deviation; CV: coefficient of variation as a percentage; Max: maximum value; Min: minimum value; Minolta parameters: L* lightness, a*: redness, b*: yellowness; CFL: centrifuge force water losses, TPA parameters: Hardness, cohesiveness, springiness, chewiness.

3.3. NIRS prediction equations interpretation

The summaries of the statistics obtained from calibration, cross-validation and external validation models in minced and intact samples are showed in Tables 2 and 3, respectively. For every parameter, the optimal number of PLS factors used was included, in order to have a lower standard error, as well as the mathematical treatments. The wavelength is not specified because the full available near infrared region was used.

As expected, coefficients of determination obtained in calibration were higher than those of external validation models for both types of samples. In the minced samples (Table 2), most of the parameters (L*, MB, shear force, hardness, cohesiveness, springiness and chewiness) showed R^2c values between 0.80 and 0.90, while R^2c values between 0.54 and 0.65 were lower for a*, b* and CFL. External validation reported lower R^2p values than calibration models, ranging from 0.70 to 0.79 for L*, MB, shear force, hardness, cohesiveness, springiness and chewiness and from 0.48 to 0.59 for a*, b* and CFL. In addition, traits with the highest R^2c in calibration were also the highest for R^2p (L*, MB, shear force, hardness, cohesiveness, springiness and chewiness).

In the NIRS analyses carried out with intact samples (Table 3), the R^2c values were higher than 0.80 only for two traits (b* and hardness) and ranged between 0.71 and 0.79 in L*, a*, MB, CFL, shear force, chewiness. Intermediate results were obtained in cohesiveness and springiness, with an R^2c value equal to 0.68. In the external validation, only hardness maintained a high R^2p value of 0.80, while the colour parameters a* and b* reached values of 0.70 and 0.73, respectively. Lastly the remaining traits had an R^2p value lower than 0.70. Comparing the two sample presentation modes, R^2 was higher in the intact than in the minced samples for four traits (a*, b* CFL and hardness). The R^2cv achieved the lowest results for all traits, both in minced and intact samples.

Regarding the residual prediction deviation (RPD), which takes into account the root-mean-square and the standard deviation of the trait, in minced samples shear force and CFL obtained RPDc values of 3.0 and 3.1, respectively, while the values were equal to or greater than 1.5 in other traits. Only the colour parameters a* and b* showed RPDc values below 1.5. In the external validation, the RPDp values were lower than those of the calibration: only shear force and cohesiveness reached a value of 1.5 while the remaining traits showed values between 1.1 and 1.4.

Again, in the analysis of intact samples (Table 3), the highest values of the statistics are clearly lower than in minced ones, since the maximum value of RPDc was 2.1 for hardness. However, RPDc values were equal to or higher than 1.5, for all the traits except for springiness. In validation, RPDp values ranged from 1.5 to 1.6 in L*, a*, b* and hardness, and between 1.0 and 1.4 in the remaining six traits. When comparing the RPD results of the two sample presentation modes, L*, a*, b*, CFL and hardness showed higher RPDp values in intact samples than in minced.

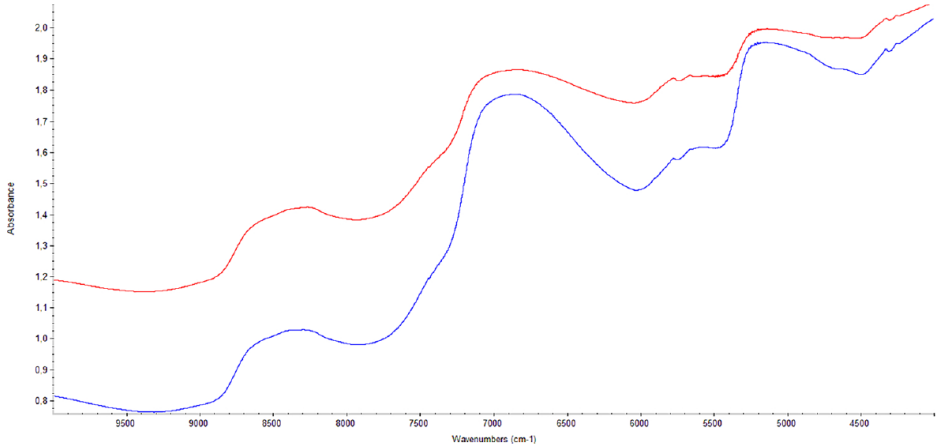


Fig. 2. Average NIR spectra of all minced (blue) and intact (red) *Longissimus thoracis et lumborum* samples of Iberian pigs fattened in an open-air free-range system (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
Legend. The Y-axis values correspond to the absorbance (1/R) of the samples along the different wavenumbers.

Table 2
Summary statistics for the PLS calibration and external validation models in minced samples.

Trait	Treatment	FPLS	Calibration				Cross - Validation			External validation				
			n	R^2c	RMSEC	RPDc	R^2cv	RMSECV	RPDcv	n	R^2p	RMSEP	RPDp	RER
L*	MSC + II deriv.+ SG filter	3	226	0.89	1.32	2.3	0.72	2.00	1.5	56	0.75	1.03	1.3	5.0
a*	MSC + II deriv. + SG filter	3	217	0.54	1.73	1.2	0.48	1.32	1.6	54	0.48	1.07	1.1	4.6
b*	MSC + II deriv. + SG filter	3	217	0.61	0.99	1.3	0.53	1.34	0.9	54	0.56	0.71	1.1	4.3
Myoglobin (mg g^{-1})	MSC + II deriv.	2	219	0.84	0.13	1.8	0.68	0.26	1.0	55	0.74	0.11	1.3	6.7
CFL (%)	MSC + II der. + SG filter.	2	216	0.65	1.39	3.0	0.49	2.80	1.5	53	0.59	1.37	1.3	4.4
Shear Force (kg cm^2)	MSC + II deriv.	2	218	0.80	0.23	3.1	0.66	0.77	0.9	55	0.70	0.44	1.9	10.6
Hardness (N)	MSC + II deriv.	2	218	0.84	1.45	1.9	0.61	2.60	1.1	55	0.74	0.99	1.3	5.2
Cohesiveness	MSC + II deriv.	3	222	0.90	0.02	2.4	0.68	0.02	1.6	56	0.79	0.02	1.5	6.1
Springiness (mm)	MSC + II deriv.	2	205	0.86	0.11	2.0	0.69	0.17	1.3	51	0.79	0.08	1.4	6.1
Chewiness (N x mm)	MSC + II deriv.	2	214	0.87	0.85	2.0	0.65	1.25	1.4	53	0.78	0.50	1.3	4.6

MSC: Multiplicative Scatter Correction; II deriv.: second derivate; SG filter: Savitzky Golay filter; Minolta parameters: L* lightness, a*: redness, b*: yellowness; CFL: centrifuge force water loss; TPA parameters: Hardness, cohesiveness, springiness, chewiness; n: number of samples; FPLS: number of PLS factors; R^2c: coefficient of determination of calibration; RMSEC: root-mean-square error of calibration; RPDc: residual prediction deviation in calibration (SD/RMSEC); R^2cv: coefficient of determination of cross-validation; RMSECV: root-mean-square error of cross-validation, RPDcv: residual prediction deviation in cross-validation (SD/RMSECV); R^2p: coefficient of determination of external validation; RMSEP: root-mean-square error of external validation; RPDp: residual prediction deviation in external validation (SD/RMSEP); RER: range error ratio in external validation.

Finally, RER advises about the suitability to categorise or quantify the samples based on the spectra of the traits. All the traits showed RER values above 4 for the minced mode (Table 2). Even shear force reached a value equal to 10.6, a result that implies a level of accuracy beyond classification and that would allow quantification with acceptable precision. Regarding the intact mode (Table 3), three traits had RER values below 4.0 (MB, cohesiveness and chewiness) and none exceeded 8.0.

Previous studies have reported a better performance of NIRS analyses in minced samples than in intact ones for moisture, protein, intramuscular fat content and fatty acid profile (Cáceres-Nevado et al., 2019; Fan et al., 2018; Ortiz et al., 2020) and for shear force (Barlocco et al., 2006). Nevertheless, to our knowledge, there are no studies that have measured myoglobin, CFL and TPA parameters in minced and intact samples. Intact muscles are heterogeneous samples with different chemical and physical characteristics due to different factors (pH, myoglobin and protein precipitation in the sarcoplasm, fibre organization, myofibrillar birefringence, sarcomere length, moisture, intramuscular fat and macroscopic surface reflectance properties) (Cozzolino et al., 2000; Cozzolino and Murray, 2002). This heterogeneity could explain a worst performance of the NIRS analyses for some traits. However, in the present study, performances of the models were better in intact samples for L*, a*, b*, CFL and hardness, since they showed higher RPDp and RER values than in minced samples (except a* for RER, which showed very similar values).

3.4. NIRS evaluation by groups of traits

3.4.1. Colour parameters

The prediction results of the MB by NIRS in intact samples were not very encouraging. RER was equal to 2.9, advising against its implementation in the conditions of this experimental design. Better results were obtained in minced meat, although somewhat contradictory: while RER was equal to 6.7, within the range of discrimination between high and low values, the RPDp value was 1.3, below the recommended for a rough screening. To our knowledge, there is no study that calibrate NIRS for MB determination on porcine meat. Prieto et al. (2006) and Ripoll

Table 3
Summary statistics for the PLS calibration and external validation models in intact samples.

Trait	Treatment	FPLS	Calibration				Cross - Validation			External validation				
			n	R^2c	RMSEC	RPDc	R^2cv	RMSECV	RPDcv	n	R^2p	RMSEP	RPDp	RER
L*	MSC + I deriv.	3	222	0.77	1.89	1.6	0.65	2.00	1.5	55	0.68	1.36	1.5	5.3
a*	MSC + I deriv.	3	230	0.79	1.27	1.6	0.66	1.55	1.3	57	0.70	1.65	1.5	4.5
b*	MSC + I deriv.	3	214	0.80	0.73	1.7	0.69	0.93	1.3	53	0.73	0.82	1.5	5.7
Myoglobin (mg g^{-1})	MSC + II deriv.	3	225	0.71	0.15	1.7	0.61	0.31	0.8	56	0.67	0.18	1.2	2.9
CFL (%)	MSC + II deriv.	4	230	0.78	2.55	1.6	0.67	2.78	1.4	57	0.69	2.14	1.4	6.8
Shear Force (kg cm^2)	MSC + II deriv.	3	198	0.73	0.45	1.6	0.68	0.97	0.8	50	0.67	0.34	1.2	4.0
Hardness (N)	MSC + II deriv.	4	226	0.88	1.28	2.1	0.66	2.00	1.3	56	0.80	1.00	1.6	5.6
Cohesiveness	MSC + II deriv.	3	195	0.68	0.03	1.5	0.53	0.04	0.9	49	0.61	0.03	1.0	3.4
Springiness (mm)	MSC + II deriv.	4	207	0.68	0.16	1.3	0.58	0.24	0.9	52	0.60	0.17	1.2	5.5
Chewiness (N x mm)	MSC + II deriv.	4	215	0.79	0.94	1.7	0.65	1.36	1.1	54	0.69	0.97	1.0	3.4

MSC: Multiplicative Scatter Correction; I deriv.: first derivate; II deriv.: second derivate; Minolta parameters: L* lightness, a*: redness, b*: yellowness; CFL: centrifuge force water losses; TPA parameters: Hardness, cohesiveness, springiness, chewiness; FPLS: number of PLS factors; n: number of samples; R^2c: coefficient of determination of calibration; RMSEC: root-mean-square error of calibration; RPDc: residual prediction deviation in calibration (SD/RMSEC); R^2cv: coefficient of determination of cross-validation; RMSECV: root-mean-square error of cross-validation, RPDcv: residual prediction deviation in cross-validation (SD/RMSECV); R^2p: coefficient of determination of external validation; RMSEP: root-mean-square error of external validation; RPDp: residual prediction deviation in external validation (SD/RMSEP); RER: range error ratio in external validation.

et al. (2008) reported RPD values of 1.09 and 2.38, respectively, in minced samples of cattle meat. The best results of Ripoll et al. (2008) were obtained working on the visible-near infrared spectrum range (400–2500 nm) and they observed a good MB estimation ability using the visible region (400–750 nm). The peak in the visible region approximately at 520 nm was probably related with the structure of meat pigments, such as myoglobin (Cozzolino et al., 2003; Cozzolino and Murray, 2002). Nevertheless, this wavenumber was not present in the near infrared region used in our study.

The observed results of RPDp in colour traits (L*, a* and b*) were higher for intact (1.5) than for minced meat (1.1–1.3), which makes its implementation suitable directly on the raw material without prior homogenization. Several authors worked on the colour parameters and their prediction by NIRS. Čandek-Potokar et al. (2006) observed slightly better results in the intact than in the minced mode in porcine *Longissimus thoracis* muscle using a similar wavelength to that used in the present study. Kapper et al. (2012b) reported better predicted statistics for L* parameter than our findings, but worse for a* and b* on porcine intact LTL muscle. Monteiro-Balage et al. (2015) showed better accuracy than ours for the three colour parameters in intact porcine LTL. This higher accuracy may be linked to the region of the electromagnetic spectrum that they used: visible (400–750 nm) and a part of the near infrared region; in fact, Monteiro-Balage et al. (2015) showed that the selection of a specific set of waveband from 405 to 690 nm plays a relevant role improving the prediction accuracy of the colour parameters. Finally, Andrés et al. (2008) and Prieto et al. (2014) reported slightly better prediction ability for L*, a* and b* in minced LTL samples of cattle trough the cross-validation procedure. This higher accuracy could be related to the electromagnetic spectrum used by Andrés et al. (2008) and Prieto et al. (2014) which also includes the region of the visible spectrum (400–2498 nm).

3.4.2. Centrifuge force loss and texture parameters

Coefficients of determination in external validation for CFL in minced and intact samples were not high (0.59 and 0.69, respectively) but RPDc was 3.0 in the minced mode, the second highest value of RPDc in the present study. However, the RPDp value decreased drastically in external validation (1.3), which was lower than the threshold necessary to categorise the samples. The best results in terms of RPDp and RER were achieved in intact mode, with a RER value of 6.8. So far, no studies are available that report results for CFL in both porcine and cattle.

The prediction results observed for shear force (measured by Warner-Bratzler) in minced samples were promising because RPD values (both in calibration and in external validation) and, above all, RER (equal to 10.6) would allow a quantification of this trait with moderate accuracy. Previous studies on cooked samples of *Longissimus thoracis* from cattle reported similar results (Ripoll et al., 2008), while other studies reported poorer prediction estimates (Prieto et al., 2008; Andrés et al., 2008). These RPD and RER statistics were clearly lower in the intact than in minced mode. Nevertheless, our results presented higher predictive ability than those described for shear force by Barlocco et al. (2006); Cai et al. (2011) and Monteiro-Balage et al. (2015) in porcine cooked samples of *Longissimus* and by Bonin et al. (2020), in cooked intact samples of *Longissimus thoracis* from cattle.

Finally, regarding TPA parameters, our results presented better prediction equations in minced samples with the exception of hardness. However, results for hardness measured in minced samples presented better predictive ability than those reported by Ripoll et al. (2008). Even if the homogenisation of minced samples could have altered the muscle structure, our results suggest that this treatment did not have a great influence on rheological traits. Cohesiveness and springiness in minced samples presented RPDp values of 1.5 and 1.4, respectively, close to the threshold required to differentiate between low and high categories, with RER equal to 6.1 which allows samples to be categorised. Hardness in intact samples reached similar results: 1.6 and 5.6 for RPDp and RER, respectively. Chewiness was the TPA characteristic with the lowest prediction ability in both modes.

3.5. NIRS predictive ability remarks

The main objective of this work was to assess NIRS methodology to simultaneously measure a set of meat quality traits, as an alternative to instrumental measurements for further implementations such as in breeding programs or in industry quality control. In addition, two presentations of the sample were included, minced and intact, seeking to simplify the process by avoiding previous sample preparations and making it more accessible on a meat production line.

The general results in terms of R^2, RMSE, RPD and RER reveals that NIRS cannot be used for an accurate and simultaneous quantification of the studied traits, as the accuracy required for strategies such as inclusion in breeding programs is not sufficient, but the results suggest the possibility of using NIRS to classify some of these traits into two or three categories. Shenk and Westerhaus (1996) set the range of R^2 of prediction models from 0.50 to 0.69 for an adequate capacity to classify samples into three classes (high, medium, and low) and from 0.70 to 0.89 for a good predictive ability. All the R^2p estimates shown in this work (except a* in minced) are within the ranges indicated by these authors. RDPp values were equal to or greater than 1.5 in six traits: shear force, cohesiveness (in minced), L*, a*, b* and hardness (in intact); RER exceed 4.0 in all traits in minced samples and in seven traits in intact (L*,

a*, b*, CFL, shear force, hardness and springiness). These values are, in both statistics, the lowest value that are considered good enough for a rough screening (Williams, 2014) or to discriminate between high and low categories (Barbin et al., 2015; Millmier et al., 2000).

The results of the present study revealed that it is not possible to obtain a simultaneous quantification of the ten traits analysed with NIRS. From an individual point of view for each trait, although the predictive models allow a rough classification in intact samples of meat colour parameters (L*, a* and b*), the measurement with the reference method using a colorimeter is even easier to implement than NIRS. The measurement of CFL in both cases (intact and minced) seems not to be accurate by NIRS, but considering that the usual analysis requires a long and laborious protocol, CFL could be indirectly measured quantifying the percentage of water losses by thawing and cooking due to their high genetic and phenotypic correlations (Fernández-Barroso et al., 2020).

Therefore, it is interesting to focus attention on MB and on texture (shear force and TPA parameters) which are not easy to quantify by traditional methods. MB is a very interesting quality trait for Iberian pigs (Ventanas et al., 2005) because consumers associate the bright cherry red colour with the high meat quality of animals raised in open-air free-range systems. However, its quantification process is long and laborious, especially when a large number of records are required. The NIRS prediction results shown here do not allow an accurate quantification of this trait. According to Cáceres-Nevado et al. (2019), the use of full spectra allows a large number of traits to be determined simultaneously. However, the region of the visible spectrum, which offers better results for MB (Prieto et al., 2006 and Ripoll et al., 2008), was not included in the considered spectral region.

Texture is also a remarkable quality trait of Iberian pigs, especially since the consumption of fresh meat has increased in the last decade. Shear force is one of the parameters related to texture, and to determinate it, a large number of replicates of the same sample have to be measured in the texturometer. Therefore, the use of NIRS instead of a texturometer would be clearly advantageous.

Tougher samples absorb more light than tender ones (Bonin et al., 2020) and this may be due to their shorter sarcomere length that allows light to penetrate easily (Andrés et al., 2007). This could explain the ability of NIRS to differentiate samples based on shear force. The results observed in this study, especially in minced samples, are very promising and encourage further research in order to improve the values of R^2, RPD and RER. Simultaneous determination of shear force and TPA parameters using NIRS would be highly advantageous, since they could replace both complex sensory analysis through trained panellists and texturometer analyses.

According to Pérez-Marín et al. (2004), both an increase in the number of samples, and an increase in trait variation, as well as an improvement of the reference methods through a more rigid standardization could be relevant factors in improving the accuracy of the NIRS calibration model. Additionally, environmental conditions such as temperature or humidity, the length of the freezing period, and grinding or centrifuging processes should be meticulously controlled to reduce small cumulative laboratory errors, which could affect the precision of the estimation using NIRS. An improvement in the quantification made with the reference methods, reducing the variability between the replicates of an individual could be a relevant factor in improving the accuracy of the NIRS calibration model.

4. Conclusion

The results of this study revealed the difficulty of a single NIRS reading, using the full spectra range, to simultaneously quantify ten meat quality traits in samples of *longissimus dorsi et lumborum* from free-range Iberian pigs. However, NIRS seems to allow the quantification of shear force in minced samples, while a rough classification is possible both of cohesiveness in minced samples and of colour parameters L*, a*, b* and hardness in intact samples. Therefore, simplification of this methodology using intact rather than minced samples was not feasible for all traits.

The possibility of applying NIRS for shear force or hardness is relevant for meat quality control since they could be incorporated into the production line, just like other analyses already implemented (*e.g.*, fatty acid profile or intramuscular fat content).

Further research is required to increase the number of samples and range of variation of the recorded traits, as well as to analyse different wavelengths for each individual trait or group of traits. The results observed for texture (shear force and TPA parameters) encourage new study designs to improve NIRS methodology for these meat quality traits, which are very relevant in the Iberian pig meat industry.

Author contributions

MA-FB participated in animal sampling, lab determinations, data analyses and drafting of the manuscript. JG-C obtained funding, participated in experimental design, animal sampling and review the manuscript. MM participated in experimental design, animal sampling and review the manuscript. P-PG contributed in lab determinations. GM and LR provided the animal material and participated in animal sampling. SP and RC contributed to NIRS calibration, bioinformatics analysis and review the manuscript. AC participated in data analyses.

Declaration of Competing Interest

All authors declare that they have not conflict of interest.

Acknowledgments

We want to thank to the company Sánchez Romero Carvajal (SRC) for the technical support. We want to thank to Wendy M. Rauw for her help revising the English style.

This work was financially supported by CON15-078, CON17-125 (INIA-SRC) and IDI- 20171141 (CDTI) grants. Miguel Ángel Fernández-Barroso was funded by FPI Ph.D. grant from the INIA institution.

References

Alberti, P., Panea, B., Ripoll, G., Sañudo, C., Olleta, J.L., Hegueruela, I., Campo, M.M., Serra, X., 2005. Medición del color. In *Estandarización de las metodologías para evaluar la calidad del producto (animal vivo, canal, carne y grasa) en los rumiantes*. Serie Ganadería Nº 3. Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid (Spain), pp. 216–225.

Alves, E., Óvilo, C., Rodríguez, M.C., Silió, L., 2003. Mitochondrial DNA sequence variation and phylogenetic relationships among Iberian pigs and other domestic and wild pig populations. Anim. Genet. 34 (5), 319–324.

Andrés, S., Murray, I., Navajas, E.A., Fisher, A.V., Lambe, N.R., Bünger, L., 2007. Prediction of sensory characteristics of lamb meat samples by near infrared reflectance spectroscopy. Meat Sci. 76, 509–516.

Andrés, S., Silva, A., Soares-Pereira, A.L., Martins, C., Bruno-Soares, A.M., Murray, I., 2008. The use of visible and near infrared reflectance spectroscopy to predict beef M. longissimus thoracis et lumborum quality attributes. Meat Sci. 78 (3), 217–224.

Barbin, D.F., Kaminishikawahara, C.M., Lourenco Soares, A., Mizubuti, I.Y., Grespan, M., Shimokomaki, M., Hirook, E.Y., 2015. Prediction of chicken quality attributes by near infrared spectroscopy. Food Chem. 168, 554–560.

Barlocco, N., Vadell, A., Ballesteros, F., Galietta, G., Cozzolino, D., 2006. Predicting intramuscular fat, moisture and Warner-Bratzler shear force in pork muscle using near infrared reflectance spectroscopy. Anim. Sci. 82, 111–116.

Barnes, R.J., Dhanoa, M.S., Lister, S.J., 1989. Standard normal variate transformation and de-trending of near infrared diffuse reflectance spectra. Appl. Spectrosc. 43 (5), 772–777.

Bonin, M., da Luz e Silva, S., Bünger, L., Ross, D., Dias Feijó, G.L., da Costa Gomes, R., Palma Rennó, F., de Almeida Santana, M., Marcondes de Rezende, F., Vinhas Ítavo, L.C., de Novais, F.J., Antonia Surita, L.M., Filgueira Pereira, M.W., Ferraz, J.B. S., 2020. Predicting the shear value and intramuscular fat in meat from Nellore cattle using Vis-NIR spectroscopy. Meat Sci. 163, 108077.

Bourne, M.C., 1978. Texture profile analysis. Food Technol. 32 (62–66), 72.

Cáceres-Nevado, J.M., Garrido-Varo, A., De Pedro-Sanz, E., Pérez-Marín, D.C., 2019. Fourier transform near-infrared spectroscopy coupled to a long fibre optic head for the quality control of IBERIAN pork loins: Intact versus minced. Meat Sci. 153, 86–93. https://doi.org/10.1016/j.meatsci.2019.03.008.

Cai, J., Chen, Q., Wan, X., Zhao, J., 2011. Determination of total volatile basic nitrogen (TVB-N) content and Warner-Bratzler shear force (WBSF) in pork using Fourier transform near infrared (FT-NIR) spectroscopy. Food Chem. 126 (3), 1354–1360.
Čandek-Potokar, M., Prevolnik, M., Skrlep, M., 2006. Ability of near infrared spectroscopy to predict pork technological traits. J. Near Infrared Spectrosc. 14 (4), 269–277.
Cozzolino, D., Murray, I., 2002. Effect of sample presentation and animal muscle species on the analysis of meat by near infrared reflectance spectroscopy. J. Near Infrared Spectrosc. 10, 37–44.
Cozzolino, D., Murray, I., Scaife, J.R., Paterson, R., 2000. Study of dissected lamb muscles by visible and near infrared reflectance spectroscopy for composition assessment. Anim. Sci. 70, 417–423.
Cozzolino, D., Barlocco, N., Vadell, A., Ballesteros, F., Gallieta, G., 2003. The use of visible and near-infrared reflectance spectroscopy to predict colour on both intact and homogenised pork muscle. LWT - Food Sci. Technol. 36, 195–202.
Estévez, M., Morcuende, D., Cava López, R., 2003. Physico-chemical characteristics of M. Longissimus dorsi from three lines of free-range reared Iberian pigs slaughtered at 90 kg live-weight and commercial pigs: a comparative study. Meat Sci. 64 (4), 499–506.
Fan, Y., Liao, Y., Cheng, F., 2018. Predicting of intramuscular fat content in pork using near infrared spectroscopy and multivariate analysis. Int. J. Food Prop. 21 (1), 1180–1189.
Fernández-Barroso, M.Á., Silió, L., Rodríguez, C., Palma-granados, P., López, A., Caraballo, C., García-Casco, J.M., Sánchez-Esquiliche, F., Gómez-Carballar, F., Muñoz, M., 2020. Genetic parameter estimation and gene association analyses for meat quality traits in open-air free-range Iberian pigs. J. Anim. Breed. Genet. 1–18. https://doi.org/10.1111/jbg.12498.
Honikel, K., 1998. Reference methods for the assessment of physical characteristics of meat. Meat Sci. 49 (4), 447–457.
Hornsey, H.C., 1956. The colour of cooked cured pork. 1. Estimation of the nitric oxide–Haem pigments. J. Sci. Food Agric. 7 (8), 534–540.
Kapper, C., Klont, R.E., Verdonk, J., Urlings, H.A.P., 2012a. Prediction of pork quality with near infrared spectroscopy (NIRS) 2. Feasibility and robustness of NIRS measurements under production plant conditions. Meat Sci. 91 (3), 300–305.
Kapper, C., Klont, R.E., Verdonk, J., Urlings, H.A.P., 2012b. Prediction of pork quality with near infrared spectroscopy (NIRS): 1 Feasibility and robustness of NIRS measurements at laboratory scale. Meat Sci. 91 (3), 294–299.
Lobos, I., Gou, P., Hube, S., Saldana, R., Alfaro, M., 2013. Evaluation of potential NIRS to predict pastures nutritive value. J. Soil Sci. Plant Nutr. 13, 463–468.
López-Bote, C.J., 1998. Sustained utilization of the Iberian pig breed. Meat Sci. 49, 17–27.
Lopez-Bote, C.J., Toldrá, F., Daza, A., Ferrer, J.M., Menoyo, D., Silió, L., Rodríguez, M.C., 2008. Effect of exercise on skeletal muscle proteolytic enzyme activity and meat quality characteristics in Iberian pigs. Meat Sci. 79 (1), 71–76.
Mayoral, A.I., Dorado, M., Guillen, M.T., Robina, A., Vivo, J.M., Vazquez, C., Ruiz, J., 1999. Development of meat and carcass quality characteristics in Iberian pigs reared outdoors. Meat Sci. 52, 315–324.
Millmier, A., Lorimor, J., Hurburgh, C., Fulhage, C., Hattey, J., Zhang, H., 2000. Near-infrared sensing of manure nutrients. Trans. ASAE 43 (4), 903–908.
Monteiro-Balage, J., da Luz, S., Abdalla Gomide, C., de Nadai Bonin, M., Figueira, A.C., 2015. Predicting pork quality using Vis/NIR spectroscopy. Meat Sci. 108, 37–43.
Ollivier, L., 2009. European pig genetic diversity: a minireview. Animal 3 (07), 915–924.
Ortiz, A., Parrini, S., Tejerina, D., Pinto de Araújo, J.P., Čandek-Potokar, M., Crovetti, A., Garcia-Casco, J.M., González, J., Hernández-García, F.I., Karolyi, D., Margeta, V., Martins, J.M., Nieto, R., Petig, M., Razmaite, V., Sirtori, F., Lebret, B., Bozzi, R., 2020. Potential use of near-infrared spectroscopy to predict fatty acid profile of meat from different European autochthonous pig breeds. Appl. Sci. 10, 5801.
Ortiz-Somovilla, V., Espana-Espana, F., Gaitan-Jurado, A.J., Perez-Aparicio, J., De Pedro-Sanz, E.J., 2006. Proximate analysis of homogenized and minced mass of pork sausages by NIRS. Food Chem. 101 (3), 1031–1040.
Osborne, B.G., 2000. Near-infrared spectroscopy in food analysis. Encyclopedia of Analytical Chemistry, pp. 1–14. https://doi.org/10.1002/9780470027318.a1018.
Parrini, S., Acciaioli, A., Crovetti, A., Bozzi, R., 2017. Use of FT-NIRS for determination of chemical components and nutritional value of natural pasture. Ital. J. Anim. Sci. 17 (1), 87–91.
Pérez-Marín, D.C., Garrido-Varo, A., Guerrero-Ginel, J.E., Gómez-Cabrera, A., 2004. Near-infrared reflectance spectroscopy (NIRS) for the mandatory labelling of compound feeding stuffs: chemical composition and open-declaration. Anim. Feed Sci. Technol. 116 (3–4), 333–349.
Pérez-Marín, D., De Pedro, E., Guerrero-Ginel, J.E., Garrido-Varo, A., 2009. A feasibility study on the use of near-infrared spectroscopy for prediction of the fatty acid profile in live Iberian pigs and carcasses. Meat Sci. 83, 627–633.
Prieto, N., Andrés, S., Giráldez, F.J., Mantecón, A.R., Lavín, P., 2006. Potential use of near infrared reflectance spectroscopy (NIRS) for the estimation of chemical composition of oxen meat samples. Meat Sci. 74 (3), 487–496.
Prieto, N., Andrés, S., Giráldez, F.J., Mantecón, A.R., Lavín, P., 2008. Ability of near infrared reflectance spectroscopy (NIRS) to estimate physical parameters of adult steers (oxen) and young cattle meat samples. Meat Sci. 79 (4), 692–699.
Prieto, N., Roehe, R., Lavín, P., Batten, G., Andrés, S., 2009. Application of near infrared reflectance spectroscopy to predict meat and meat products quality: a review. Meat Sci. 83, 175–186.
Prieto, N., López-Campos, Ó., Aalhus, J.L., Dugan, M.E.R., Juárez, M., Uttaro, B., 2014. Use of near infrared spectroscopy for estimating meat chemical composition, quality traits and fatty acid content from cattle fed sunflower or flaxseed. Meat Sci. 98 (2), 279–288.
Prieto, N., Juárez, M., Larsen, I.L., López-Campos, Ó., Zijlstra, R.T., Aalhus, J.L., 2015. Rapid discrimination of enhanced quality pork by visible and near infrared spectroscopy. Meat Sci. 110, 76–84.
Ripoll, G., Albertí, P., Panea, B., Olleta, J.L., Sañudo, C., 2008. Near-infrared reflectance spectroscopy for predicting chemical, instrumental and sensory quality of beef. Meat Sci. 80 (3), 697–702.
Sammut, C., Webb, G.I., 2011. Leave-one-out cross-validation. Encyclopedia of Machine Learning. Springer, Boston, MA. https://doi.org/10.1007/978-0-387-30164-8_469.
SanCristobal, M., Chevalet, C., Haley, C.S., Joosten, R., Rattink, A.P., Harlizius, B., Groenen, M.A., Amigues, Y., Boscher, M.Y., Russell, G., Law, A., Davoli, R., Russo, V., Désautés, C., Alderson, L., Fimland, E., Bagga, M., Delgado, J.V., Vega-Pla, J.L., Martinez, A.M., Ramos, M., Glodek, P., Meyer, J.N., Gandini, G.C., Matassino, D., Plastow, G.S., Siggens, K.W., Laval, G., Archibald, A.L., Milan, D., Hammond, K., Cardellino, R., 2006. Genetic diversity within and between European pig breeds using microsatellite markers. Anim. Genet. 37 (3), 189–198.
Savenije, B., Geesink, G.H., Van Der Palen, J.G.P., Hemke, G., Hopkins, D., Ouali, A., 2006. Prediction of pork quality using visible/near-infrared reflectance spectroscopy. Meat Sci. 73 (1), 181–184.
Shenk, J., Westerhaus, M., 1996. Calibration the ISI Way. In: Davis, A.M.C., Williams, P. (Eds.), Near Infrared Spectroscopy: The Future Waves, NIR Publications, pp. 198–202. Chichester (U.K.).
Solís, M., De Pedro, E., Garrido, A., García, J., Silió, L., Rodríguez, C., Rodrigañez, J., 2001. Evaluación de la composición del lomo de cerdo ibérico mediante la tecnología NIRS. In: Zaragoza (Spain)Proceedings of the Asociación Interprofesional para el Desarrollo Agrario – ITEA, II, pp. 613–615.
Tejerina, D., García-Torres, S., Cava, R., 2012. Water-Holding capacity and instrumental texture properties of M. Longissimus Dorsi and M. Serratus Ventralis from Iberian Pigs as affected by the production system. Livest. Sci. 148 (1–2), 46–51.
Tejerina, D., García-Torres, S., Cabeza de Vaca, M., Ortiz, A., Romero-Fernández, M.P., 2018. Evaluation of near-infrared spectroscopy (NIRS) for the quality control of packaged cured ham-sliced from Iberian pigs. Archivos de Zootecnia. Proceedings IX Simposio Internacional sobre el Cerdo Mediterráneo 231–234. https://doi.org/10.21071/az.v67iSupplement.3611.
Ventanas, S., Ventanas, J., Ruiz, J., Estévez, M., 2005. Iberian pigs for the development of high-quality cured products. Proc. Recent Res. Develop. Agric. Food Chem. 6 (27–53). Research Signpost, Kerala, (India).
Wang, W., Peng, Y., Sun, H., Zheng, X., Wei, W., 2018. Real-time inspection of pork quality attributes using dual-band spectroscopy. J. Food Eng. 237, 103–109.
Williams, P., 2014. The RPD statistic: a tutorial note. NIR News 25, 22–26.
Williams, P.C., Sobering, D.C., 1993. Comparison of commercial near infrared transmittance and reflectance instruments for analysis of whole grains and seeds. J. Near Infrared Spectrosc. 1, 25–32.
Zamora-Rojas, E., Pérez-Marín, D., De Pedro-Sanz, E., Guerrero-Ginel, J.E., Garrido-Varo, A., 2012. In-situ Iberian pig carcass classification using a micro-electro-mechanical system (MEMS)-based near infrared (NIR) spectrometer. Meat Sci. 90, 636–642.
Zamora-Rojas, E., Garrido-Varo, A., De Pedro-Sanz, E., Guerrero-Ginel, J.E., Pérez-Marín, D., 2013. Prediction of fatty acid content in pig adipose tissue by near infrared spectroscopy: at-line versus in-situ analysis. Meat Sci. 95 (3), 503–511.

CAPÍTULO III

Differences in loin tenderness of Iberian pigs explained through dissimilarities in their transcriptome expression profile.

Fernández-Barroso M.A., Caraballo C., Silió L., Rodríguez C., Núñez Y., Sánchez-Esquiliche F., Matos G., García-Casco J.M. & Muñoz M. 2020. Animals 10 (9), 1715; https://doi.org/10.3390/ani10091715.

animals

MDPI

Article

Differences in the Loin Tenderness of Iberian Pigs Explained through Dissimilarities in Their Transcriptome Expression Profile

Miguel Ángel Fernández-Barroso [1,2,*], Carmen Caraballo [1,2], Luis Silió [2], Carmen Rodríguez [2], Yolanda Nuñez [2], Fernando Sánchez-Esquiliche [3], Gema Matos [3], Juan María García-Casco [1,2] and María Muñoz [2]

1 Centro Nacional de I+D del Cerdo Ibérico (CENIDCI), INIA, 06300 Zafra, Spain; carmen.caraballo@inia.es (C.C.); garcia.juan@inia.es (J.M.G.-C.)
2 Departamento de Mejora Genética Animal, INIA, 28040 Madrid, Spain; luis.siliolopez@gmail.com (L.S.); mrodriguezvaldovinos@gmail.com (C.R.); nunez.yolanda@inia.es (Y.N.); mariamm@inia.es (M.M.)
3 Sánchez Romero Carvajal—Jabugo, SRC, 21290 Huelva, Spain; fernando.sanchez@osborne.es (F.S.-E.); gema.matos@osborne.es (G.M.)
* Correspondence: fernandez.miguel.inia@gmail.com

Received: 11 August 2020; Accepted: 18 September 2020; Published: 22 September 2020

Simple Summary: The Iberian pig is the most representative autochthonous breed of the Mediterranean region with unique genetic and phenotypic characteristics. The breed has been successfully preserved by its high-quality meat and high-priced products. Tenderness is one of the most relevant meat quality traits, and meat tenderization is influenced by genetic and environmental effects such as pre-slaughter handling and post-mortem conditions. Tenderness could be included in Iberian pig breeding programs, mainly focused on the improvement of premium-cuts percentage, in order to avoid the meat quality decline. A better biological understanding of this trait is needed. In the current study, we analyze the transcriptome of pigs divergent for Warner–Bratzler shear force through RNA-seq technique for the identification, characterization and quantification of candidate genes involved in biological pathways, networks and functions affecting meat tenderness.

Abstract: Tenderness is one of the most important meat quality traits and it can be measured through shear force with the Warner–Bratzler test. In the current study, we use the RNA-seq technique to analyze the transcriptome of *Longissimus dorsi* (LD) muscle in two groups of Iberian pigs (Tough and Tender) divergent for shear force breeding values. We identified 200 annotated differentially expressed genes (DEGs) and 245 newly predicted isoforms. The RNAseq expression results of 10 genes were validated with quantitative PCR (qPCR). Functional analyses showed an enrichment of DE genes in biological processes related to proteolysis (*CTSC*, *RHOD*, *MYH8*, *ACTC1*, *GADD45B*, *CASQ2*, *CHRNA9* and *ANKRD1*), skeletal muscle tissue development (*ANKRD1*, *DMD*, *FOS* and *MSTN*), lipid metabolism (*FABP3* and *PPARGC1A*) and collagen metabolism (*COL14A1*). The upstream analysis revealed a total of 11 transcription regulatory factors that could regulate the expression of some DEGs. Among them, IGF1, VGLL3 and PPARG can be highlighted since they regulate the expression of genes involved in biological pathways that could affect tenderness. The experiment revealed a set of candidate genes and regulatory factors suggestive to search polymorphisms that could be incorporated in a breeding program for improving meat tenderness.

Keywords: RNA-seq; transcriptome analysis; Iberian pig; meat tenderness

1. Introduction

Traditionally, the meat industry and genetic breeding programs have been focused on production traits such as efficient growth rate and carcass leanness. However, an intensive selection for them could alter some porcine muscle characteristics [1] and quality traits [2]. Furthermore, the antagonistic correlation among pigs selected for lean muscle and body growth versus tenderness has been reported [3]. Moreover, muscle from pigs intensively selected for increased lean growth showed lower tenderness [4]. Meat quality plays a key role in determining its commercial value and consumer acceptance, tenderness being one of its most appreciated characteristics.

Meat tenderness is a complex trait influenced by the interaction of many effects, such as genotype, gene expression, environmental conditions, pre-slaughter handling, slaughter and *post-mortem* procedures [5]. Tenderness is moderately heritable, with values ranging from 0.25 to 0.45 both in commercial and autochthonous pig breeds [6–8]. In addition, several polymorphisms in candidate genes such as *Calpastatine* (*CAST*) or *Calpain 1* (*CAPN1*) affecting tenderness have been identified [8–10]. Its measurement is not easy, the instrumental texture analysis by Warner–Bratzler shear force being one of the most common methods since it is considered an objective and rapid approach [11,12].

One extended approach for identifying candidate genes harboring potential mutations that could partially explain the genetic basis of a particular trait consists in analyzing expression gene changes between individuals divergent for the studied trait. High-throughput RNA sequencing technique (RNA-seq) permits the identification, characterization and quantification of the transcript dataset expressed in any tissue. Previous transcriptome studies using RNA-seq for sequencing the muscle transcriptome of different pig breeds and crossbreds have reported some interesting information about gene expression, biological pathways, networks and functions related with tenderness [13–15].

The Iberian pig is the most representative autochthonous breed belonging to the Mediterranean region. This breed is characterized by its high adipogenic potential, voracious appetite, high protein turnover ratio and low lean tissue deposition [16] that are determined by their traditional open-air production system [17] and its unique genetics characteristics [18–20]. These features mean that both their fresh meat and derived dry-cured products are vastly appreciated, obtaining a high economic value in Spanish and international markets. The inclusion of different quality traits in the objectives of these programs may be required. The use of molecular genetics techniques is therefore advisable and it has been previously approached in other studies [8,21].

Taking this into account, in the current study we analyze the transcriptome of *Longissimus dorsi* (LD) muscle in Iberian pigs divergent for shear force to identify differentially expressed genes (DEGs) and understand how they are involved in the regulation of biological processes. Therefore, the aims of this study were: (a) to identify and quantify the DEGs and recognize biological processes, pathways, networks and functions in which these genes are involved, (b) to determine transcription regulatory factors influencing the observed gene expression profile, and (c) to propose a set of candidate genes with detected mutations affecting meat tenderness.

2. Materials and Methods

2.1. Animal Material and Phenotypic Data

The animals used in the current study were commercial castrated male pigs that belonged to an Iberian purebred line closed for approximately 15 years described in a previous study [8]. Animals were fattened in an open-air free-range system (*Montanera*) based on ad libitum intake of acorns and grass. They were managed during three successive years (from 2015 to 2017), being slaughtered at an approximate age of 17 months and with an average slaughter weight of 165 kg. Animal handling was carried out according to the regulations of the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63/EU about the protection of animals used in research. Protocols were assessed and approved by the INIA Committee of Ethics in Animal Research, which is the named Institutional Animal Care and Use Committee (IACUC) for the INIA.

Longissimus dorsi samples from 892 animals were removed from the carcass after slaughter. A central muscle section of approximately 300 g were separated of each loin for meat determination. These samples were vacuum-packed in nylon/polyethylene bags and stored at −20 °C until analysis. After that, samples were thawed and subsequently cooked by immersion at 70 °C during 1 h in a water bath (VWR, Pennsylvania, USA) [22]. Texture was determined in cooked meat portions following [11] and measured as cooked meat shear force (SFF) by the Warner–Bratzler test (Stable Microsystems TA.XT Plus, Godalming, UK). Eight pieces of 3 cm × 1 cm × 1 cm (length, width and thickness) were cut perpendicular to the muscle fiber direction with a Warner–Bratzler blade (HDP/BSW) and the eight repeated measures were averaged. SFF was measured as kg/cm^2. The mean of the SFF was 4.33 (SD = 1.10).

The following mixed model was used to estimate breeding values (EBVs) for SFF:

$$y = Xb + Za + Wsm + e \quad (1)$$

where **y** is the vector of SFF values corresponding to each animal, **b** represents the vectors of systematic effects in which the slaughter weight was fitted as a covariate, **a** is the vector of the additive genetic effects (EBVs) distributed as N (0, $\boldsymbol{A}\sigma^2_u$), where $\boldsymbol{A}$ is the numerator of kinship matrix that allows for the adjustment of the data taking into account the pedigree information, **sm** is the vector of the combined fattening-slaughter batch environmental random effects (19 levels) and **e** the vector including the residual effects. **X, Z** and **W** are the incidence matrices. EBVs were estimated using the TM program [23].

A total of 13 pigs with the most extreme EBVs for SFF, avoiding full and half siblings, were selected. The Tough group contained six individuals showing the highest EBVs, and the Tender group contained the seven ones with the lowest values. The EBV averages were 2.11 (SD = 0.29) and −1.03 (SD = 0.05) for the Tough and the Tender group, respectively, and the corresponding phenotypic mean values for shear force were 9.17 kg/cm^2 (SD = 1.28) and 2.83 kg/cm^2 (SD = 0.48).

2.2. Transcriptomic Analyses

2.2.1. RNA Extraction, Library Preparation and Sequencing

The individual samples of *Longissimus dorsi* collected after slaughter were introduced on cryogenic tubes, frozen in liquid nitrogen and stored at −80 °C until analysis. RiboPure TM of High-Quality total RNA kit (Ambion, Austin, TX, USA) was used to extract the total RNA, following the manufacturer's recommendations. RNA was quantified using a NanoDrop equipment (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer device (Agilent Technologies, Santa Clara, CA, USA) was employed to evaluate the RNA integrity (RNA Integrity Number = RIN), the RIN values obtained for all the samples were in the range from 7 to 8.

Paired-end libraries were built using TruSeq SBS Kit v3 (Illumina, San Diego, CA, USA) for each sample. Multiplex sequencing of the libraries was carried out on a HiSeq2000 sequence analyzer (Illumina, Inc, San Diego, CA, USA) with four samples per lane at Centro Nacional de Análisis Genómico (CNAG-CRG; Barcelona, Spain), according to the manufacturer's instructions. Pair-end reads of 74 bp were generated. The raw sequence data of 12 of the 13 individuals have been deposited in the Gene Expression Omnibus (GEO) database with the accession number: GSE155915. The sample named Tender_ 7 in the present study was already sequenced in a previous study [24] and its sequence data was already deposited in GEO with the accession number GSE116951 and its identification corresponds to the individual H5.

2.2.2. Bioinformatics Analyses

Mapping, Assembly and Identification of Novel Isoforms

The quality of raw sequencing data was evaluated with FastQC (Babraham Bioinformatics, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The quality parameters measured with this tool corresponds to sequence-read lengths and base-coverage, nucleotide contributions and base ambiguities, quality scores and over-represented sequences. All the samples passed the quality control (QC) parameters: same length, 100% coverage in all bases, 25% of A, T, G and C nucleotide contributions, 50% GC on base content and less than 0.1% of overrepresented sequences. TrimGalore was used to trim the raw sequences through removing the sequencing adaptor and poly A and T tails, setting default values (stringency of 6 bp) and keeping paired-end reads when both pairs were longer than 40 bp. TopHat v2.1.0 [25] was used to map the filtered reads against the pig reference genome (Sscrofa11.1). Cufflinks v2.2.1 [26] was employed to assemble and quantified the transcripts in fragments per kilobase of transcript per million (FPKM) mapped reads. The normalized expression data have been deposited in the GEO database with the accession number GSE155915 and GSE116951. Cuffcompare tool (from Cufflinks) was used to identify isoforms not described so far. It was run using Ensembl (Sscrofa11.1) transcriptome annotation as a reference to assess the accuracy of the predicted Cufflinks mRNAs or gene models. Finally, a list with all class codes of the transcript was reported by Cuffcompare.

Differential Expression Analyses

Cuffdiff was used to quantify the expression values and carry out the differential expression analyses between the Tough and Tender groups of annotated genes and novel described isoforms. Cuffdiff was run setting the bias correction (-b option) and the rescue method for multireads (-u option). The remaining parameters were established as default. These genes and novels isoforms were filtered according to the following criteria: an average group expression greater than 0.5 FPKM and a fold change value (FC) of the expression differences between the Tender and Tough groups' ≤ 0.67 and ≥ 1.5. Besides, R package q-value [27] was used to correct multiplicity of test, where q-value provides a method to control the false discovery rate (FDR), which is the proportion of false positives among all positive results, and genes and new isoforms were considered as differentially expressed with a p-value ≤ 0.05 and q-value ≤ 0.10.

Gene Functional Classification, Network and Pathway Analyses

Gene Ontology (GO) information was used to analyze the functionality of the DEGs between the Tough and Tender groups. The biological interpretation of the data was carried out using FatiGO browser from Babelomics 5 (Babelomics 5, http://babelomics.bioinfo.cipf.es/). The potential interactions between the proteins codified by the DEGs and clustering through the Markov Cluster Algorithm (MCL) were studied using STRING tools v11.0 [28].

Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Qiagen, CA, USA) bioinformatics tool was used to identify and characterize biological functions, gene networks, canonical pathways and transcription regulatory factors affected by the DEGs. This software assesses the significant association between the data set of DEGs and canonical pathways. In addition, it builds networks with the set of genes using the records harbored in the Ingenuity Pathways Knowledge Base. Potential regulators of differential gene expression were also identified using the tools "upstream regulators" and "causal networks"; these tools analyze if the potential transcriptional factors and upstream regulators contained in the Ingenuity Knowledge Base repository activate or inhibit the differential gene expression pattern through the estimation of a z-score. This z-score statistically measures the significance between the regulator and its potential targets and the direction among them [29].

2.3. RNA-seq Results Validation by Quantitative PCR

RNA from the 13 animals used in RNA-seq study was used to perform the technical validation of the RNA-seq experiment through measuring the expression of 10 genes (*MSTN*, *ANKRD1*, *ACTC1*, *MX1*, *FOS*, *COL1A1*, *ELOVL6*, *SSH2*, *NOS2*, *and IRF1*) with quantitative PCR (qPCR). Six genes were selected from de list of DEGs (upregulated in the Tough group or in the Tender group) and four were not differentially expressed between the Tough and Tender (two of them showed low expression and the other two showed a medium-high expression). In a first step, first-strand cDNA synthesis was carried out using Superscript II (Invitrogen, Life Technologies, Paisley, UK) and random hexamers in a total volume of 20 µL using 1 µg of total RNA, according to the manufacturer's instructions.

Primer pairs used for quantification were designed using Primer-Blast (NCBI, https://www.ncbi.nlm.nih.gov/tools/primer-blast/) from the available GENBANK and/or Ensembl sequences, covering different exons in order to assure the amplification of the cDNA. Table S1 shows primer sequences and amplicon lengths. Then, a standard PCR on cDNA for each primer was carried out to verify amplicon sizes. Next, following standard procedures, SYBR Green Mix (Roche, Basel, Switzerland) in a LightCycler480 (Roche, Basel, Switzerland) was used for the quantification, and data analysis was carried out with LightCycler480 SW1.5 software (Roche, Basel, Switzerland). Three technical replicates were run per each sample and dissociation curves were obtained to confirm the specific amplification of each gene. A total of four cDNA dilutions were carried out in order to build a standard curve and estimate PCR efficiency. Mean crossing point values (Cp) were used for performing the statistical analyses. The Cp value is the PCR cycle number at which the sample's reaction curve intersects the threshold line. Genorm software was used to calculate the stability of the endogenous genes *ACTB* and *B2M* [30] and these endogenous genes were used to normalize the data through normalization factors. Relative quantities of DEGs were divided by normalization factors, which were the geometric means of the two reference gene quantities. Finally, the technical validation was performed studying the Pearson correlation between the expression values obtained from RNA-Seq data (FPKM) and the normalized gene expression data obtained by qPCR and calculating the concordance correlation coefficient (CCC) [31] between fold change values estimated from RNA-Seq and qPCR expression measures by the two techniques for the 10 genes.

3. Results

3.1. Characterization of Longissimus Dorsi Transcriptome

In the present study, the *Longissimus dorsi* tissue transcriptome of 13 animals was characterized with the RNA-seq technique. All samples passed the quality control. We obtained 1474 million raw paired-end reads and 1457 million reads after trimming and filtering. A range from 92.80% to 94.50% of the reads mapped to the porcine reference genome was used (Table S2). These results agree with the previous study carried out in the same tissue of pigs from the same population but in different individuals (except L7) [24] and with a study carried out in *Semimembranosus* muscle tissue on Large White pigs [32].

Table S3 shows the classification of the transcripts in relation to the Ensembl annotated porcine genes and a total of 109,085 transcripts expressed in the 13 animals were detected by Cufflinks tool. The potentially new isoforms annotated represents a 43.29% of the transcripts. The intergenic transcripts predicted were a 7.14% of the total and the percentage of transcripts falling entirely within a reference intron was a 14.88%, this could be related with intron retention events, incorrect annotation of exons, errors or missing prediction of isoforms [33].

Expression distribution values of the 25,878 genes annotated in the pig genome reported with Cuffdiff are shown in Figure S1, where the distribution of gene expression levels in FPKMs was similar for the Tough and Tender groups.

3.2. Differential Expression Analyses

The differential expression analyses revealed a total of 200 annotated genes and 245 newly predicted isoforms differentially expressed between the Tough and Tender groups. A total of 118 annotated genes were upregulated in the Tender group (FC ≤ 0.67) while 82 genes were upregulated in the Tough group (FC ≥ 1.5) (Table S4). Besides, 128 newly predicted isoforms presented higher expression in the Tender group and 117 in the Tough group. Regarding the fold change, values ranged from 0.04 to 8.83. The genes with the highest expression differences between groups were *GBP1* (*FC* = 0.09, *p*-value = 5×10^{-5}, overexpressed in the Tender group) and *FAM180B* (FC = 8.83, *p*-value = 3.5×10^{-4}, overexpressed in the Tough group) (Table 1). The further functional analyses were focused on differentially expressed annotated genes.

Table 1. Fold change, mean expression value in the Tender and Tough groups, *q-value* and with *p-value* < *0.05*, corresponding to the most relevant differentially expressed genes.

Gene	Fold Change	Tender	Tough	*q*-Value
Guanylate binding protein 1 (GBP1)	0.089	46.959	4.175	0.002
Cholinergic Receptor Nicotinic Alpha 9 Subunit (CHRNA9)	0.360	1.666	0.600	0.002
Ras Homolog Family Member D (RHOD)	0.438	1.414	0.620	0.025
Calsequestrin 2 (CASQ2)	0.447	15.284	6.832	0.002
Ankyrin Repeat Domain 1 protein (ANKRD1)	0.476	553.625	263.267	0.002
Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha (PPARGC1A)	0.508	13.825	7.024	0.015
Cathepsine C (CTSC)	0.567	63.679	36.137	0.016
Fatty Acid Binding Protein 3 (FABP3)	0.633	352.059	222.898	0.038
Fos proto-oncogene (FOS)	1.670	24.722	41.282	0.037
Dystrophin (DMD)	1.779	0.614	1.091	0.038
Collagen Type XIV Alpha 1 Chain (COL14A1)	2.017	3.035	6.122	0.057
Myostatin (MSTN)	2.038	2.977	6.067	0.009
Growth Arrest and DNA Damage Inducible Beta protein (GADD45B)	2.871	12.954	37.188	0.002
Actin Alpha 1, Skeletal Muscle (ACTC1)	4.085	9.750	39.830	0.002
Myosin Heavy Chain 8 (MYH8)	7.541	1269.750	9574.930	0.002
Family With Sequence Similarity 180 Member B (FAM180B)	8.830	1.959	17.297	0.008

Mean expression values are expressed in fragments per kilobase of transcript per million mapped fragments (FPKMs).

3.3. Gene Functional Analysis

The GO enrichment analyses carried out with FatiGO identified 457 GO biological processes (GO_{BP}) and two GO_{SLIM} (cut-down versions of the GO ontologies containing a subset of the terms in GO) enriched in DEGs (Table S5). Table 2 shows a summary of significant overrepresented pathways that could be more related with meat tenderness. For instance, these pathways are involved in skeletal muscle tissue development (GO: 0007519, 10 genes), regulation of muscle system process (GO: 0090257, seven genes), collagen metabolic process (GO: 0032963, six genes), regulation of calcium ion transport (GO: 0051924: six genes), c-Jun N-terminal kinase (JNK) cascade (GO: 0007254, six genes), actin-myosin

filament sliding (GO: 0033275, four genes), skeletal muscle tissue growth (GO: 0048630, two genes), positive regulation of proteolysis involved in cellular protein catabolic process (GO: 1903052, four genes) and actomyosin structure organization (GO: 0031032, three genes).

Table 2. Summary of the most relevant significantly overrepresented Gene Ontology (GO) terms related with tenderness on differentially expressed genes (DEGs) using FatiGO.

Term	Genes	Adjusted *p*-Value
GO_{BP}		
Skeletal muscle tissue development (GO:0007519)	*MYLK2, MSTN, FOS, HLF, CXCL10, IGFBP5, ANKRD1, DMD, CXCL9, FOXN2*	1.02×10^{-9}
Muscle cell development (GO:0055001)	*CXCL10, ANKRD1, DMD, CXCL9, CASQ2, ACTC1, COL14A1*	9.94×10^{-6}
Skeletal muscle cell differentiation (GO:0035914)	*MYLK2, FOS, HLF, ANKRD1, FOXN2*	9.94×10^{-6}
Regulation of muscle system process (GO:0090257)	*MYLK2, MSTN, CTGF, DMD, ADRA2C, CASQ2, COL14A1*	3.72×10^{-5}
Collagen metabolic process (GO:0032963)	*CTGF, COL1A2, COL1A1, ENG, COL12A1, COL14A1*	3.72×10^{-5}
Response to amino acid (GO:0043200)	*CTGF, COL1A2, COL1A1, CDO1, PPARGC1A*	4.07×10^{-5}
Cytosolic calcium ion transport (GO:0060401)	*CTGF, CXCL10, DMD, CXCL9, THY1, CASQ2*	6.75×10^{-5}
Regulation of muscle tissue development (GO:1901861)	*MSTN, PPARGC1A, CXCL10, CXCL9, COL14A1*	3.70×10^{-4}
Regulation of calcium ion transport (GO:0051924)	*CXCL10, DMD, CXCL9, ATP2B2, THY1, CASQ2*	4.40×10^{-4}
Actin-myosin filament sliding (GO:0033275)	*MYLK2, MYH8, DMD, ACTC1*	4.53×10^{-4}
JNK cascade (GO:0007254)	*CTGF, SFRP4, PAK1, TRIB1, DUSP10, GADD45B*	4.53×10^{-4}
Negative regulation of protein kinase activity (GO:0006469)	*PPP1R1B, THY1, DUSP1, TRIB1, DUSP10, GADD45B*	5.19×10^{-4}
Collagen fibril organization (GO:0030199)	*COL1A2, COL1A1, COL12A1, COL14A1*	1.33×10^{-4}
Actin-mediated cell contraction (GO:0070252)	*MYLK2, MYH8, DMD, ACTC1*	1.11×10^{-3}
Regulation of muscle contraction (GO:0006937)	*MYLK2, CTGF, DMD, ADRA2C, CASQ2*	1.24×10^{-3}
Regulation of JNK cascade (GO:0046328)	*CTGF, SFRP4, PAK1, DUSP10, GADD45B*	1.27×10^{-3}
Regulation of stress-activated MAPK cascade (GO:0032872)	*CTGF, SFRP4, PAK1, DUSP10, GADD45B*	2.26×10^{-3}
Regulation of stress-activated protein kinase signaling cascade (GO:0070302)	*CTGF, SFRP4, PAK1, DUSP10, GADD45B*	2.27×10^{-3}
Skeletal muscle tissue growth (GO:0048630)	*MSTN, IGFBP5*	2.97×10^{-3}
Regulation of protein kinase B signaling (GO:0051896)	*SLC9A3R1, ITSN1, IGFBP5, RASD2*	3.48×10^{-3}
Actin filament bundle organization (GO:0061572)	*RHOD, CTGF, PAK1, PFN2*	4.82×10^{-3}
Positive regulation of proteolysis involved in cellular protein catabolic process (GO:1903052)	*ZFAND2A, CTSC, TRIB1*	7.85×10^{-3}
Actomyosin structure organization (GO:0031032)	*ANKRD1, CASQ2, ACTC1*	1.61×10^{-2}

Subsequently, STRING tools v11.0 revealed networks of protein–protein interactions codified by annotated DEGs and novel predicted isoforms (Figure S2). Five differentiated clusters were observed, two clusters comprised proteins codified by DEGs overexpressed in the Tender group (clusters 1 and 2) and three clusters comprised proteins codified by DEGs upregulated in the Tough group (clusters

3, 4 and 5). Cluster 1 constituted TAP1, PSMB8, PSMB9, TNFRSF12A and SPOPL associated with the cellular amino acid metabolic process and protein and peptide regulations. Cluster 2 constituted CXCL9, CXCL10, ADRA2C, CCL4, CTSC, FCN2, C2, C3, C4 and C1QA associated with skeletal muscle tissue development, calcium ion transport and proteolysis regulation. Cluster 3 constituted COL1A1, COL1A2, COL12A1 and COL14A1 associated with cellular and collagen metabolic processes. Cluster 4 constituted MYLK2, MYLK4 and PAK1 associated with skeletal muscle tissue development and protein autophosphorylation. Cluster 5 constituted MSTN, DMD and AQP4 associated with skeletal muscle tissue development.

Furthermore, functional analysis carried out with IPA software revealed 12 networks enriched in DEGs (Table S6). In these analyses, the networks were ranked according to their size and the number of targeted genes and a network score was assigned. This score is estimated as the negative logarithm of the *p*-value calculated by Fisher's exact test. The two networks closely related with tenderness and muscle development are showed in Table 3. Functions described in gene network #5 (Figure 1) are related with *connective tissue development and function, tissue morphology and lipid metabolism* and in gene network #6 (Figure 2) with *cell morphology, cellular assembly, organization, function and maintenance.* Other networks which enclosed relevant DEGS among the Tender and Tough groups are represented in Figure S3 (network #8) and Figure S4 (network #9).

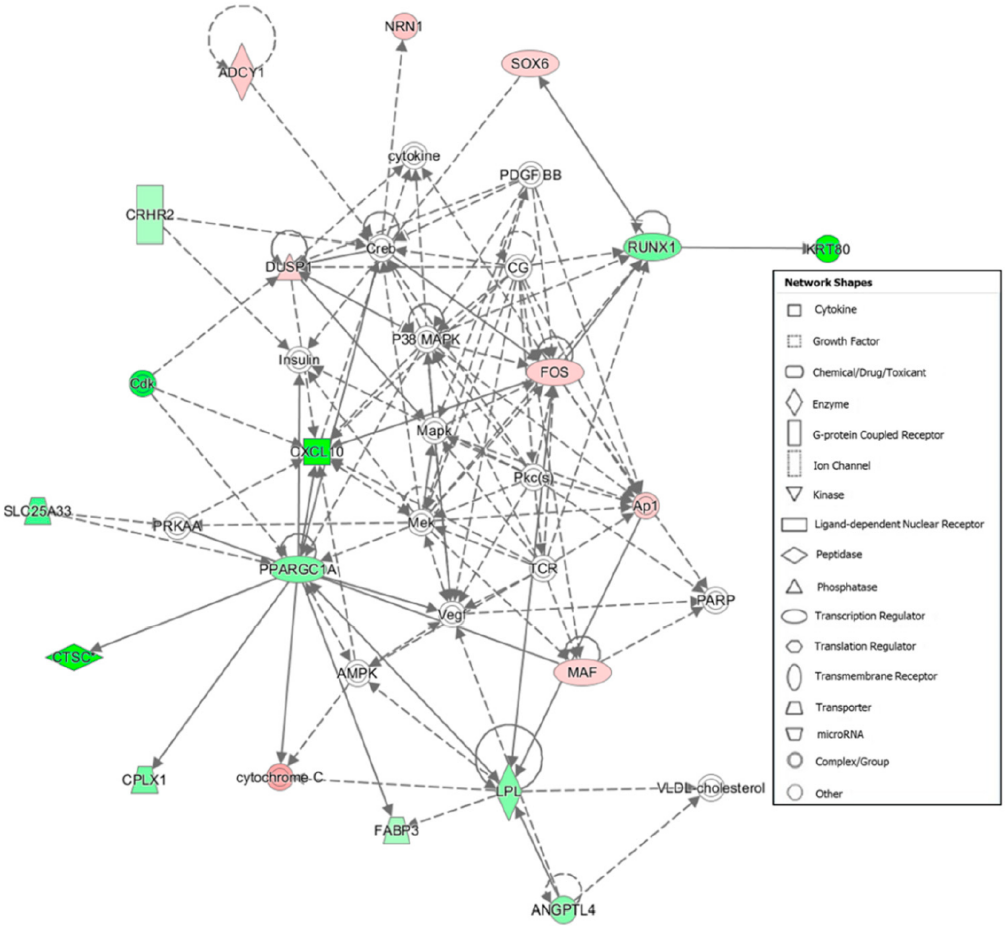


Figure 1. Gene network #5: Connective Tissue Development and Function, Lipid Metabolism, Tissue Morphology. Genes up-regulated and down-regulated in the Tender group are represented in green and red colors, respectively.

Table 3. List of relevant enriched networks and functions related with tenderness identified in the set of DEGs between the Tender and Tough groups identified by IPA software. Genes showing the highest expression differences between groups are in bold.

ID	Molecules in Network	Score	Focus Molecules	Functions
5	ADCY1, AMPK, ANGPTL4, Ap1, Cdk, CG, CPLX1, Creb, CRHR2, CTSC, CXCL10, cytochrome C, cytokine, DUSP1, FABP3, FOS, Insulin, KRT80, LPL, MAF, Mapk, Mek, NRN1, P38 MAPK, PARP, PDGF BB, Pkc(s), PPARGC1A, PRKAA, RUNX1, SLC25A33, SOX6, TCR, Vegf, VLDL-cholesterol	26	17	Connective Tissue Development and Function, Lipid Metabolism, Tissue Morphology
6	ACTC1, AHSP, AQP4, ARPP21, BAZ1A, CCL4, CD3, CHRNA10, CHRNA9, CRABP2, DMD, ERK, FLNC, FSH, GK, Histone h3, Histone h4, HLF, IgG, Jnk, Lh, miR-130a-3p (and other miRNAs w/seed AGUGCAA), mir-672, MYH8, MYLK2, Nr1h, RNA polymerase II, SLC9A7, Smad2/3, Sos, THY1, TMEM184A, TRIB1, WFDC1, ZIC1	23	16	Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance

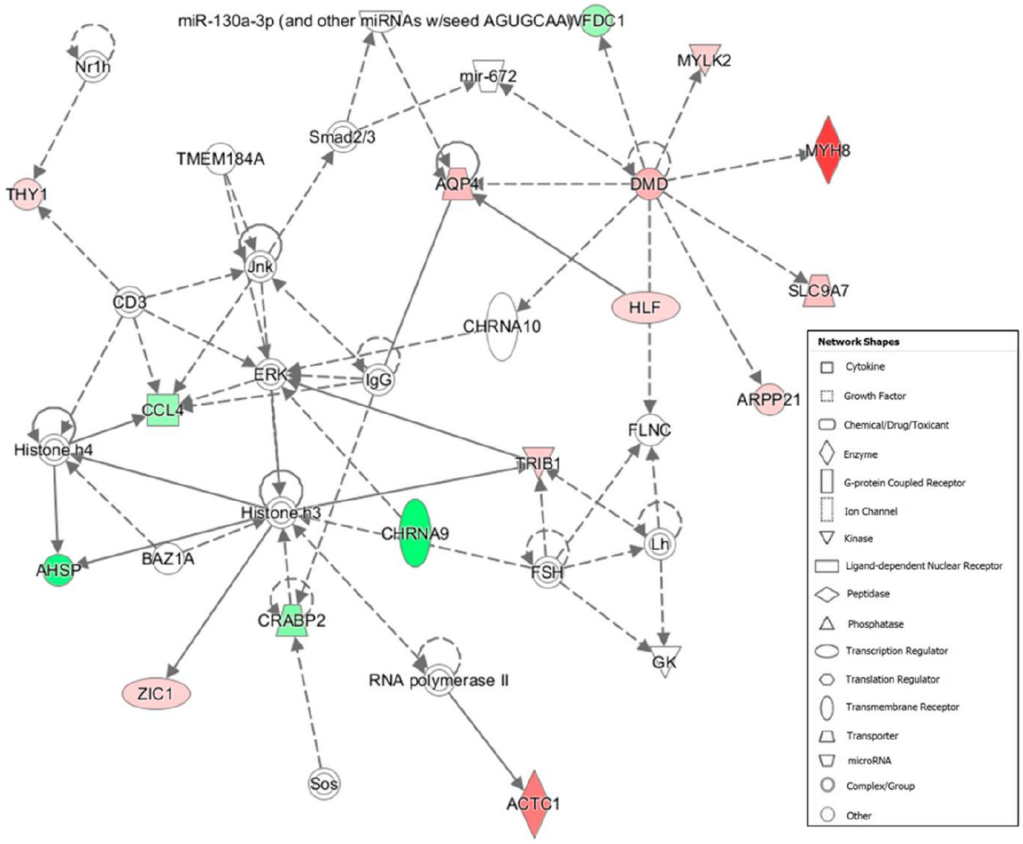


Figure 2. Gene network #6: Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance. Genes up-regulated and down-regulated in the Tender group are represented in green and red colors, respectively.

3.3.1. Canonical Pathways Analysis

An additional functional interpretation of global gene expression differences was carried out. A total of 86 canonical pathways were significantly enriched (p-value < 0.05) in the dataset of 200 DEGs (Table S7). Furthermore, the analysis reported 12 pathways with assigned z-score, predicting an overall increase in the activity of the pathway in the Tough group when z-score was greater than zero and an overall increase of the pathway in the Tender group when z-score was less than zero, but none of them were significantly activated or inhibited (z-score > 2 or < −2, Table 4). For instance, RhoA Signaling, PPARα/RXRα Activation and White Adipose Tissue Browning showed a trend for activation in the Tender group. On the other hand, some of the pathways presented a positive z-score indicating a trend for activation in the Tough group, as Actin Cytoskeleton Signaling, ILK signaling, Tec Kinase Signaling, Integrin Signaling and Rho Family GTPases. With regards to other significantly enriched canonical pathways (p-value < 0.05, Table S7), it is worth mentioning calcium signaling (p-value = 0.02) and IGF-1 signaling (p-value = 8.7×10^{-3}).

We found some evidence that canonical pathways related to cell cycle, motility, organization and function, apoptosis, immunological system and lipid metabolism were enriched in the Tender group and that pathways related to skeletal muscle development and growth, such as with cell function, movement and survival presented a trend for activation in the Tough group.

Table 4. List of significant pathways (p-value < 0.05) with assigned z-score identified in the set of DEGs according to the Tender and Tough group identified by Ingenuity Pathway Analysis (IPA) software.

Canonical Pathways	p-Value	Ratio	z-Score	Molecules
Integrin Signaling	0.001	0.033	0.447	ACTC1, MYLK2, PAK1, PFN2, RASD2, RHOBTB1, RHOD
Hepatic Fibrosis Signaling Pathway	0.001	0.027	1.265	CCN2, COL1A1, COL1A2, FOS, MYLK2, RASD2, RHOBTB1, RHOD, TFRC, YAP1
Actin Cytoskeleton Signaling	0.002	0.032	1.342	ACTC1, FN1, MYH8, MYLK2, PAK1, PFN2, RASD2
Synaptogenesis Signaling Pathway	0.003	0.026	−0.378	ADCY1, ADCY6, CPLX1, ITSN1, MARCKS, PAK1, RASD2, SNCG
PPARα/RXRα Activation	0.004	0.032	−1.342	ADCY1, ADCY6, GPD2, LPL, PPARGC1A, RASD2
ILK Signaling	0.004	0.032	1.342	ACTC1, FN1, FOS, MYH8, RHOBTB1, RHOD
Tec Kinase Signaling	0.009	0.031	1	ACTC1, FOS, PAK1, RHOBTB1, RHOD
Cardiac Hypertrophy Signaling	0.011	0.025	−1	ADCY1, ADCY6, ADRA2C, RASD2, RHOBTB1, RHOD
GNRH Signaling	0.011	0.029	1	ADCY1, ADCY6, FOS, PAK1, RASD2
Signaling by Rho Family GTPases	0.012	0.025	0.447	ACTC1, CIT, FOS, PAK1, RHOBTB1, RHOD
RhoA Signaling	0.015	0.033	−1	ACTC1, CIT, MYLK2, PFN2
White Adipose Tissue Browning Pathway	0.018	0.031	−1	ADCY1, ADCY6, LDHB, PPARGC1A

Ratio: number of DEGs in a pathway divided by the number of genes comprised in the same pathway.

3.3.2. Transcription Regulatory Factors

The upstream analysis and regulator effect tools of IPA were applied to analyze potential transcription regulatory factors of DEGs involved in different molecular processes, which may explain the differential expression observed between the Tender and Tough group.

A total of 860 transcriptional regulators were identified (p-value < 0.05, Table S8). Moreover, the sense of activation state was statistically significant predicted for 11 of them (z-score > 2 or z-score < −2, Table 5), five were activated in the Tender group (z-score < −2, KLF11, IL4, PPARG, OGT and NOS2) and six were activated in the Tough group (z-score >2, IGF1, VGLL3, SEMA7A, PTH, TRIM24 and SATB1). Regulator effect tool predicted just one regulator effect network (Figure 3). This network represented a causal hypothesis to interpret the regulatory potential mechanism of the upstream regulator (IGF1) in the expression of some DEGs.

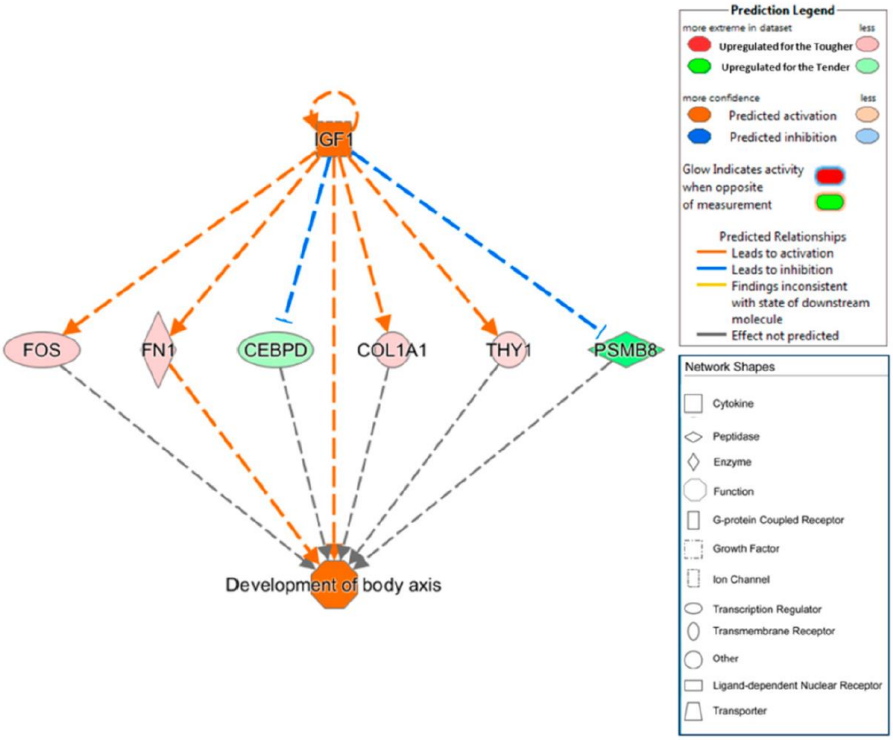


Figure 3. Regulator effects network predicted as activated in purebred Iberian pigs fattened in a free-range system. In the upper tier is *IGF1* (predicted to be activated, orange color). In the middle, there are the genes whose expression changes in response to the activation of *IGF1* (green upregulated for the Tender group and red upregulated for the Tough group). Dashed lines between *IGF1* and DEGs represent the interactions, predicted to be activated (orange lines) or predicted to be inhibited (blue lines). In the lower tier, the expected phenotypic activate function (development of body axis, orange color) is shown.

Table 5. List of significant upstream regulators identified in the set of DEGs according to the Tender and Tough group (p-value < 0.05 and z-score > 2 or < −2).

Upstream Regulator	Molecule Type	PAS	Activation z-Score	p-Value of Overlap	Molecules in Dataset	Related Functions
IGF1	Growth factor	Activated	2.947	7.29×10^{-8}	CCL4, CEBPD, COL1A1, DUSP1, FN1, FOS, IGFBP5, LPL, MYH8, PSMB8	Development of body axis
VGLL3	Other	Activated	2.000	1.99×10^{-6}	COL12A1, COL1A1, COL1A2, GADD45B	
SEMA7A	Transmembrane receptor	Activated	2.000	3.31×10^{-5}	CCN2, COL1A1, COL1A2, FN1	
PTH	Other	Activated	2.197	4.51×10^{-5}	COL1A1, COL1A2, DUSP1, FOS, IGFBP5, SFRP4	
KLF11	Transcription regulator	Inhibited	−2.236	4.92×10^{-4}	CCN2, COL1A2, CPT2, ENG, FABP3, PPARGC1A	
IL4	Cytokine	Inhibited	−2.331	5.93×10^{-4}	ALDOC, CCL26, CCL4, CD163, CXCL10, FOS, LPL, NABP1, PPARGC1A, TFRC	

Table 5. *Cont.*

Upstream Regulator	Molecule Type	PAS	Activation z-Score	*p*-Value of Overlap	Molecules in Dataset	Related Functions
TRIM24	Transcription regulator	Activated	2.236	1.30×10^{-3}	CXCL10, PSMB10, PSMB8, PSMB9, TAP1	
PPARG	Ligand-dependent nuclear receptor	Inhibited	−2.179	1.89×10^{-3}	ANGPTL4, COL1A1, COL1A2, CPT2, CRABP2, FABP3, FN1, IGFBP5, LPL, PPARGC1A	
OGT	Enzyme	Inhibited	−2.000	2.42×10^{-3}	FOS, LPL, PPARGC1A, THY1	
NOS2	Enzyme	Inhibited	−2.219	3.13×10^{-3}	ACTC1, CCL4, CTSC, CYCS, PPARGC1A, THY1	
SATB1	Transcription regulator	Activated	2.000	4.18×10^{-2}	GADD45B, HBB, MAF, RUNX1	

PAS: Predicted Activation State, predicted Activated in the Tough group (z-Score > 2), predicted Inhibited in the Tough group (z-Score < −2).

3.4. RNA-Seq Validation by qPCR

The relative expression of 10 genes was quantified with qPCR in the 13 samples in order to validate the results observed in the RNA-seq technique. We calculated Pearson correlation between RNA-seq and qPCR expression values, their corresponding *p*-values and the CCC. Table 6 shows the results of technical validation, where seven of the total of genes presented a correlation coefficient > 0.7, nine genes showed a significant *p*-value (*p*-value < 0.05) and only *MSTN* gene presented a suggestive significance value (*p*-value = 0.06). The CCC was equal to 0.828, suggesting a substantial general concordance between RNA-seq and qPCR expression values [31]. In addition, the *IRF1* gene showed the highest agreement between methods and *MSTN* gene presented the lowest concordance.

Table 6. Technical validation of RNA-seq results by quantitative PCR (qPCR): Fold Change values (FC), Pearson correlations (r^2) and Concordance Correlation Coefficient (CCC) between expression values obtained from both techniques.

Gene	Expression Type	qPCR FC	RNAseq FC	r^2	*p*-Value	CCC
ACTC1	Tender < Tough	2.861	4.085	0.986	6.20×10^{-10}	0.828
MX1	Tender < Tough	2.231	2.195	0.943	1.34×10^{-6}	
COL1A1	Tender < Tough	1.503	1.636	0.922	7.29×10^{-6}	
FOS	Tender < Tough	1.634	1.670	0.913	1.29×10^{-5}	
ANKRD1	Tender > Tough	0.531	0.476	0.779	0.002	
MSTN	Tender < Tough	1.753	2.038	0.527	0.064	
IRF1	NO DE	0.656	0.235	0.997	1.81×10^{-13}	
NOS2	NO DE	0.611	0.811	0.701	0.008	
SSH2	NO DE	1.037	1.625	0.584	0.036	
ELOVL6	NO DE	0.887	1.712	0.565	0.044	

NO DE: No differentially expressed in RNA-seq experiment. Tender > Tough: higher expression in the Tender than in the Tough group. Tender < Tough: lower expression in the Tender than in the Tough group.

4. Discussion

In the present study, functional analysis of DEGs revealed a set of biological processes, canonical pathways and networks, potentially related with tenderness. In the functional enrichment analyses using FatiGO, there were an overrepresentation of processes related with *Proteolysis* such as Positive regulation of proteolysis involved in cellular protein catabolic process (GO:1903052), Cytosolic calcium ion transport (GO:0060401), Regulation of calcium ion transport (GO:0051924) or Actin-myosin filament

sliding (GO:0033275); *Skeletal muscle tissue development and growth*: Skeletal muscle tissue development GO:0007519), Muscle cell development (GO:0055001), Skeletal muscle cell differentiation (GO:0035914), or Regulation of muscle system process (GO:0090257); *Lipid metabolism*: Lipid homeostasis (GO:0055088), Lipid storage (GO:0019915) or Positive regulation of lipid storage (GO:0010884) and *Collagen metabolic process*: Collagen metabolic process (GO:0032963), Collagen fibril organization (GO:0030199) or Collagen biosynthetic process (GO:0032964) (Table 2 and Table S5).

Next, the most relevant DEGs and their potential implications in the aforementioned biological pathways and processes will be detailed.

4.1. Proteolysis Process

It is well-known that the proteolytic system has a key role in meat tenderization [34], which is related with the degree of post-mortem alteration of proteins and muscle structure [35]. Several proteases such as calpains, calpastatins, cathepsins, caspases and kinases are involved in the meat tenderization process [34]. During the conversion of muscle to meat, cathepsins degrade actomyosin binding [34] and the weakening of the strong actomyosin interaction imply the widening of sarcomeres. Then, calpains are more able to hydrolyze associated proteins, allowing proteolysis and influencing the maturation of muscle [36]. In our study, a higher expression of the *Cathepsine C* gene (*CTSC*) in the Tender group was observed (Table 1). The functional analyses revealed that this gene is associated with the positive regulation of proteolysis (GO:1903052, Table 2). In a study comparing muscle expression in the Casertana pig breed with two commercial breeds, an overexpression of *CTSC* in Casertana muscle was also observed [37]. Like the Iberian breed, Casertana is an autochthonous breed characterized for having better meat quality than commercial ones. These results support that higher expression of *CTSC* is associated with a higher activation of the proteolysis process favoring the meat tenderization. Besides, in a variant calling analyses based on RNA-seq data of two Polish pig breeds divergent for meat tenderness, variants with different genotype distribution between breeds on *CTSC* gene were detected [38]; however, any association analyses between the genetic variants identified and tenderness have been carried out so far.

Furthermore, *Ras Homolog Family Member D (RHOD)* is overexpressed in the Tender group and codifies for a protein involved in reorganization of the actin cytoskeleton. Our functional analysis showed that *RHOD* was involved on actin filament organization GO_{BP} (GO: 0061572) (Table 2). *RHOD* gene maps on the porcine chromosome 2 (5.39 Mb) within a quantitative trait loci (QTL) for shear force detected in the F2 of a Duroc x Pietrain crossbred [39]. In vitro studies have revealed that the interference of RHOD protein produces a higher cell attachment and diminishes cell migration [40]. Therefore, higher expression of the *RHOD* gene could ease the degradation of actin cytoskeleton during proteolysis.

Two of the most overexpressed genes in the Tough group were *Myosin Heavy Chain 8 (MYH8)* and *Actin Alpha 1, Skeletal Muscle (ACTC1)*, both enclosed in functional network #6 (Table 3), and functional analysis revealed that *MYH8* and *ACTC1* play a relevant role in GO_{BP} as actin-myosin filament sliding, structure organization and contraction (Table 2) and *ACTC1* is also involved in muscle cell development (GO: 0055001). MYH8 protein is related with functions as skeletal muscle contraction, ATPase activity ([41] and actin filament binding [42]. A higher expression of this gene was related with muscle hypertrophy in a transcriptome analysis on Canadian double-muscled Large White pigs, which are characterized by having a notable muscle mass [43]. *ACTC1* encodes for a protein involved in skeletal muscle development [44] and contributes to the structural integrity of cytoskeleton [45]. Expression differences of *ACTC1* associated to tenderness have been uneven. In a study comparing the transcriptome of *Longissimus dorsi* between Shaziling pig, an autochthonous Chinese pig breed with a high-quality meat than Yorkshire, an overexpression of *ACTC1* was observed in Shaziling pig [46]. However, the study was carried out in 25-day-old pigs and the results could be different in older animals. On the other hand, in a study comparing the *Longissimus dorsi* transcriptome of male and female Qinchuan cattle individuals, in which females have tenderer meats, a down-regulation of

ACTC1 gene was observed. In our study, the overexpression of *ACTC1* is apparently associated with tougher meat.

GADD45B gene was overexpressed in the Tough group. This gene encodes for Growth Arrest and DNA Damage Inducible Beta protein, which plays a crucial role in cellular growth arrest and apoptosis, associated with stress signals [47]. The authors of [48] observed a higher expression of *GADD45B* in cattle *Longissimus thoracis* muscle with high ultimate pH values. Alteration of pH implies changes in the regulation of calcium transport pathways into the cellular sarcoplasm. When pH muscle is at isoelectric point (5.2 to 5.5) an increase in calcium concentration in the cell is produced, causing a rise of calpain activity [49,50], which degrades myofibrillar and cytoskeletal proteins, promoting meat tenderization [51]. A disparity of results regarding the relationship between pH and tenderness has been reported by other authors. While [6] did not observe a phenotypic relation between these traits, [52] determined that the relationship between pH and tenderness depends on the breed. In this study, we do not have pH values and we cannot conclude that the differential expression of *GADD45B* gene among groups could be explained by the pH.

ACTC1 and *RHOD* codify for proteins involved on ILK and Integrin signaling pathways (Table 4). ILK Signaling is related with cell survival and apoptosis [53] and Integrin Signaling is linked with cell apoptosis and regulation of actin cytoskeleton [54]. Interestingly, [15] reported pathways involved in cellular apoptosis (survival) and stress response as important factors of tenderization. Moreover, apoptosis is considered one of the first steps in development of meat tenderization, inducing biochemical and structural muscle changes [55]. In the same direction that *GADD45B, ACTC1* and *RHOD* have been associated with cellular apoptosis.

Other important DEGs that could be involved in proteolysis, with higher expression in the Tender group, were *Calsequestrin 2* (*CASQ2*), *Cholinergic Receptor Nicotinic Alpha 9 Subunit* (*CHRNA9)* and *Ankyrin Repeat Domain 1 protein* (*ANKRD1*). *CASQ2* codifies for a protein involved in calcium store in the sarcoplasmic reticulum and also modulate calcium homeostasis, calcium release and muscle contraction [56]. In the functional analyses, there was an enrichment of the *CASQ2* gene in GO annotations related with calcium transport and muscle contraction (Table 2). Differential expression of *CASQ2* was also observed in several studies contrasting the transcriptome of breeds divergent for several meat quality parameters including tenderness in some cases. These studies compared the muscle transcriptome of Basque vs. Large White [13], Iberian vs. Duroc × Iberian crossbred [57] and Wannanhua vs. Yorkshire breeds [58]. However, in these studies, the highest expression level of *CASQ2* was observed in the breed with the tougher meats. This disagreement could be due to the fact that the expression differences observed in these studies are between breeds divergent for different quality traits and that, in our study, we analyzed the expression differences between Iberian pigs divergent for meat tenderness.

ANKRD1 gene was proposed as candidate gene for meat quality by [59] since they observed in their study that it could be a transcriptional regulator of myogenesis and of myofibril assembly in porcine LD muscle of Duroc x Pietrain. In the present study, an enrichment of this gene was observed in GO_{BP} related with cellular assembly involved in morphogenesis and myofibril assembly, actomyosin structure organization and sarcomere organization (Table S5). In addition, *ANKRD1* is involved in biological processes related with muscular growth as skeletal muscle tissue development and muscle cell differentiation linked to myogenesis (Table 2). The authors of [13] also observed a higher expression of *ANKRD1* in Large White than in Basque pigs and proposed that ANKRD1 interacts with CASQ2 protein, which regulates calcium homeostasis in skeletal muscle as it was observed in cardiac muscle [56]. The overexpression of both genes in tenderer meat group observed in our study would support this hypothesis.

*Cholinergic Receptor Nicotinic Alpha 9 Subuni*t (*CHRNA9*) was enriched in a biological process related to the regulation of cytosolic calcium concentration (Table S5), and the canonical pathway analysis interpreted that *CHRNA9* is involved in Calcium signaling pathway, together with *ACTC1*, *CASQ2* and *MYH8* genes (Table S7). High expression of *CHRNA9* was associated with tenderer meats

in F2 animals from Duroc × Pietrain cross [60]. One more time, a regulation of the calcium releasing to the cytoplasm would have related with proteolytic enzymatic activity and have an influence on meat tenderness.

4.2. Skeletal Muscle Tissue Development and Growth

As we mentioned above, the genetic selection of most common European breeds has usually been focused on improving the efficiency of lean tissue growth. The increase in growth rate and lean meat percentage could alter other meat characteristics such as myofiber composition [61] which would have an impact on meat tenderness. Nevertheless, it should be noted that Iberian pigs have not been previously selected for this or other related traits.

Our transcriptome analysis revealed some pivotal DEGs related with cellular and muscle development such as *MSTN*, *DMD*, and *FOS*, were overexpressed in the Tough group. *Myostatin* (*MSTN)* encodes for a protein that inhibits myogenesis. This process consists of the growth and differentiation of muscle. The inhibition or loss of function of this gene produces an increase in muscle and reduced fat mass that have been reported in several animal species as cattle [62] or sheep [63]. In pigs, *MSTN* null mutations generated in Meishan individuals reproduced the double muscle phenotype and meat from pigs homozygous for the mutation was tenderer than the wild-type ones [64]. This study agrees with our results, and both seem to be contradictory since we would expect that animals with higher muscle mass have tougher meat. However, the role paper of MSTN on adipogenesis has to be considered too. Despite this general lower fat mass content, an inhibition of adipogenesis in intramuscular preadipocytes isolated from porcine *Longissimus dorsi* muscles has been observed [65]. In the current study, the intramuscular fat content in the animals with tougher meat was lower (%IMF = 3.38 ± 0.73) than that measured in the group with tenderer meat (%IMF = 7.81 ± 2.29). Therefore, this higher expression in the Tough group could inhibit the adipogenesis in intramuscular fat of these animals.

DMD encodes for dystrophin protein, which has a relevant role in structural function stabilizing the sarcolemma and anchoring the extracellular matrix to the cytoskeleton via F-actin [66]. The authors of [67] suggested that a decrease in the activity of this essential protein may result in progressive porcine *Biceps femoris* muscle degeneration and wasting. Network #6 (Figure 2) shows that *DMD* could activate *MYH8*. Therefore, higher expression of *DMD* seems to result in a better assembly of actin filament binding, which could be more resistant to degradation by proteases hindering the meat tenderization.

Fos proto-oncogene (*FOS*) belongs to the immediate early gene family of transcription factors. *FOS* is involved in the maintenance of cytoskeleton, cell-grown regulation, proliferation and differentiation [68]. *FOS* gene maps in a QTL for skeletal muscle fiber detected in a Meishan x Pietrain F2 [69] and codifies for a transcription factor involved that has been previously identified as regulating myogenesis [70]. Differential expression of this gene on muscle has been observed between different breeds divergent for growth and meat quality at different age stages [13,71,72]. In the current study, functional analyses related this gene with skeletal muscle tissue development and cell differentiation (Table 2) as well as connective tissue development (Figure 1). Moreover, IPA analysis showed that *FOS* participates on the IGF-1 signaling pathway (Table S7), which is involved in the activation of receptor tyrosine kinase activity, thereby initiating cell proliferation, cell differentiation and cell survival [73,74] also is an important regulator of cellular growth and metabolism [73].

4.3. Lipid Metabolism

It is well known that the intramuscular fat (IMF) content is a main determinant of tenderness in pig. The positive relation between IMF and tenderness could be due to fat cell expansion that may open the muscle structure favoring the muscle separation [75]. However, this relationship is controversial, and it is very influenced by the pig breed [52]. Both IMF and tenderness are heritable traits and the positive genetic correlation among them suggests a common genetic background between IMF and tenderness has also been reported by several authors [76,77]. In addition, some transcriptome studies

have shown that genes encoding proteins implicated in IMF accretion are overexpressed in tender pork [78] and it was also proposed that higher IMF content could ease the tenderization associated with the cooking process [78].

Interestingly, our results showed some DEGs involved in biological process related with lipid metabolism that are overexpressed in animals with tenderer meat such as *Fatty Acid Binding Protein 3 (FABP3)* and *Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha (PPARGC1A)*. *FABP3* encodes for a member of the fatty acid-binding protein family that comprises a group of small cytosolic proteins, which specifically bind and transport intracellular fatty acids. There are several studies that find associations between polymorphisms in *FABP3* gene and IMF in different pig breeds [79–81]. Besides, [80] reported associations between polymorphisms mapped in the *FABP3* gene and tenderness, and a positive correlation between the expression of this gene and IMF in muscle of a Korean x Yorkshire F2. *PPARGC1A* codifies for a transcription factor which regulates hormone receptors and transcription factors involved in adipogenesis and adipocyte differentiation [82] also promotes the fiber conversion to oxidative-type ones [83]. Therefore, this protein could be related with tenderness not only favoring the adipogenesis and IMF content but also for its influence in muscle fiber composition. Actually, there are several studies that report association between polymorphisms located in this gene and tenderness in a commercial hybrid pig population [84]. In the current study, Figure 1 shows as *PPARGC1A* activates *FABP3* and *CTSC* that could suggest favoring the adipogenesis and proteolysis in the group with tenderer meat. Furthermore, *PPARGC1A* is involved in PPARα/RXRα Activation and White Adipose Tissue Browning pathways, which presented a trend for activation in the Tender group and are related with lipid metabolism. Peroxisome proliferator-activated receptor-α (PPARα) heterodimerizes with retinoid x receptor (RXR) and play a role in the transcription of regulator genes of adipocyte differentiation and fatty acid oxidation [85].

It is well known that there is a moderate antagonism between muscular development and intramuscular fat content (IMF) in pigs [86]. In the previous study carried out in the same pig population [24], an overexpression of genes related with myogenesis and skeletal muscle development on animals with low IMF content such as *ACTC1*, *DMD* and *FOS* was observed. As we previously mentioned, our findings indicate that *ACTC1*, *DMD* and *FOS* are upregulated in the Tough group, supporting the hypothesis that IMF is related to the tenderization process in Iberian pigs.

4.4. Collagen Metabolic Process

Collagen protein determines the structural support and strength of the extracellular matrix in the connective tissue [87]. Collagen content depends on animal species and age. For instance, collagen crosslinks in older animals is considered related with tougher meat [88] and meat tenderness usually decreases when animals are older as well. Therefore, collagen content seems to contribute to meat toughness. High correlation between collagen content and shear force values measured with Warner–Bratzler method on cattle were found [89]. However, other studies have observed lower correlations in different cattle breeds and ages [90,91]. In a theoretical study, [92] revealed that meat can be ranked in terms of tenderness using the number of collagen crosslink per volume of cooked meat.

In our study, we detected higher expression of several collagen-encoding genes in tougher meat samples compared with tenderer samples, suggesting a differentiation in collagen constituents between divergent samples for shear force. Our gene ontology analysis revealed that cluster 3 (Figure S2) contained DEGs from the collagen family (*COL1A1*, *COL1A2*, *COL12A1* and *COL14A1*), upregulated in the Tough group. Among these DEGs cited previously, *Collagen Type XIV Alpha 1 Chain* (*COL14A1*) encodes for a protein that plays a key role in the extracellular matrix structure organization, cell-cell adhesion and collagen fibril organization [93]. Other authors have also reported differential expression of *COL14A1* between pigs that, a priori, can be divergent for meat tenderness. In the same sense, here, [57] showed that *COL14A1* was upregulated in the transcriptome of Duroc x Iberian pigs compared with Iberian purebred pigs, which are expected to have tenderer meat. In addition, higher expression

of this gene was observed in Yorkshire pigs than in Wannanhua [58] and Wei [94] pig breeds with better meat quality properties.

In summary, the use of two different bioinformatics software for functional analysis showed that some of the most significant differential expressed genes encode proteins that have been involved in similar relevant biological functions, networks and pathways. Genes encoding for proteins involved in proteolysis and activators of the conversion of muscle to meat in *post-mortem* process are overexpressed in tenderer meat. Otherwise, those genes codifying for proteins that activate myogenesis, stimulate the muscle development and constitute the extracellular matrix of connective tissue are overexpressed in tougher meat.

The results here are very relevant and support that part of the tenderness variability can be explained by genetics. However, tenderness is a complex trait that can be affected by pre-slaughter conditions as stress situations and other post-mortem factors as temperature [1]. These factors should also be always controlled to avoid undesirable meat textures.

4.5. Transcription Regulatory Factors

A study of the potential regulatory factors explaining the observed expression differences between groups was also carried out. It is not necessary that the regulatory factors are differentially expressed since they can join to DNA sequences adjacent to DEGs with more or less affinity due to potential mutations located in these DNA motifs or in coding sequences of the regulatory factors that could alter the final protein structure.

The IPA analyses predicted a regulator effect network that could explain the expression of some DEGs. Figure 3 represented causal hypotheses to interpret the regulatory potential mechanism of the upstream regulator IGF1 on *FOS, FN1, COL1A1* and *THY1*. Apparently, IGF1 activates the expression of *FOS, FN1, COL1A1* and *THY1* that are overexpressed in Iberian pigs with tougher meats and inhibits the expression of CCAAT/enhancer binding protein delta (*CEBPD*) and proteasome subunit beta 8 (*PSMB8*) which are repressed in this type of pigs. As was explained above, *FOS* is involved in muscle growth and development and *COL1A1* in the extracellular matrix constitution; therefore, a higher activation of this process seems to make tougher meat. On the other hand, *CEBPD* plays an essential role during the earliest phases of the adipocyte differentiation [95] and *PSMB8* maps in a genomic region explaining part of the IMF phenotypical variance observed in Iberian pigs [96]. Therefore, IGF1 would activate the muscle growth and inhibit adipogenesis explaining the antagonism relationship between these traits.

One of the most significant regulatory factors is vestigial-like family member 3 (VGLL3) (Table 5), which was identified as a transcriptional co-factor associated with myogenesis, skeletal muscle development and muscle hypertrophy [97]. VGLL3 was predicted to be activated in tougher samples inducing the expression of *COL12A1, COL1A1, COL1A2* and *GADD45B* genes. Some of them are involved in collagen metabolic process (*COL12A1, COL1A1*, and *COL1A2*), cellular growth and apoptosis (*GADD45B*). Therefore, a higher activity of this transcription factor would hinder the tenderization process.

Peroxisome proliferator-activated receptor gamma (PPARG) is a ligand-dependent nuclear receptor known as the "master regulator of adipogenesis", being related with lipid metabolism processes as adipose differentiation [98] and it has been identified as a potential candidate genes for improving IMF content [99]. Moreover, a higher expression of PPARG gene have been observed in Iberian piglets [100] and foetuses [72] than in Duroc x Iberian piglets and Large-White foetuses, respectively, which have less IMF content than Iberian ones. In this study, although the expression differences of this gene were not observed, the upstream analysis identified this gene as an activator of *FABP3* and *PPARGC1A*, which, as has been pointed out above, are overexpressed in the Tender group and promote adipogenesis and increase IMF content.

4.6. Candidate Genes for an Iberian Pig-Breeding Program

The ultimate objective of this study was to propose several candidate genes for searching polymorphisms and design a genotyping panel for improving tenderness in Iberian pigs. In further steps, polymorphisms with divergent allelic frequencies should be identified in the regulatory regions of the proposed candidate genes and association analyses between their genotypes and shear force should be carried out in the same Iberian pig population.

In summary, the most promising candidate genes to be selected are involved in proteolysis processes (*ACTC1, ANKRD1, CHRNA9, CTSC* and *RHOD*), skeletal muscle tissue development and growth (*DMD* and *FOS*), lipid metabolism (*FABP3* and *PPARGC1A*) and collagen metabolic process (*COL14A1*). Although the *MSTN* gene is clearly involved in muscle growth, the results observed here are controversial since this protein inhibits myogenesis and a higher expression in the Tough group was observed; therefore, more cautions should be taken before to be included as a selection marker.

It is also interesting to consider genes encoding regulator factors such as *IGF1*, *PPARG* and *VGLL3* since they modulate the expression of some of the genes mentioned before.

It is worth mentioning that some genes, such as *ACTC1, DMD* and *FOS*, were also overexpressed in Iberian pigs with low IMF content (Muñoz et al., 2018); therefore, they could be used for improving both IMF content as shear force (tenderness).

5. Conclusions

In our study, we identified 200 differentially expressed annotated genes and 245 newly predicted isoforms on the LD muscle transcriptome of 13 Iberian pigs with divergent breeding values for tenderness measured through data of shear force with Warner–Bratzler analysis. The use of two different pieces of bioinformatics software for the functional analysis of these DEGs has revealed relevant biological processes, canonical pathways and networks potentially related with tenderness. The most representative functions associated with this trait are proteolysis, skeletal muscle development, lipid metabolism and collagen metabolism. Generally, genes encoding for proteins involved in proteolysis and conversion of muscle to meat (*ANKRD1*, *CASQ2*, *CHRNA9*, *CTSC*, and *RHOD*) are overexpressed in the Tender group while genes encoding for proteins enhancing myogenesis and muscle development (*FOS* and *DMD*) are overexpressed in the Tough one. In addition to this, genes involved in lipid (*FABP3* and *PPARGC1A*) and collagen metabolisms (*COL14A1*) are also relevant. Additionally, the upstream analysis has identified several transcriptional regulatory factors (IGF1, PPARG and VGLL3) that regulate the expression of some differentially expressed genes mentioned before, such as *FOS* or *COL1A1.*

This study is a first approach to understand the biological mechanisms underlying the trait meat tenderness and it provides a set of candidate genes that could harbor polymorphisms affecting tenderness in Iberian pigs. However, further studies including functional analyses such as immunohistochemical staining and/or Western blot analysis should be performed to experimentally validate if the proteins codified by the proposed candidate genes are responsible of the variation in tenderness. Additional steps, such as identifying polymorphisms with opposed allelic frequencies in the extreme groups, performing association analyses between the identified polymorphisms and tenderness and assessing their effects on other quality meat and productive traits, should be carried out to apply this information in a breeding program to improve tenderness in the Iberian pig.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/10/9/1715/s1, Table S1: Primer name, sequences, melting temperature (TM) and amplicon size of genes selected for qPCR validation, Table S2: Total number of reads, filtered reads, and percentage of mapped reads per sample, Table S3: Classification of the transcripts identified in the *Longissimus dorsi* muscle of Iberian pigs in relation to the Ensembl annotated pig genes, Table S4: Fold change, mean expression value in the Tender and Tough groups, *p*-value, and *q*-value corresponding to DEGs and differentially expressed novel isoforms, Table S5: List of overrepresented GO terms on DEGs between the Tender and Tough groups using FatiGO, Table S6: Complete list of networks, diseases and functions identified on DEGs between the Tender and Tough groups by IPA software, Table S7: Ingenuity Pathway Analysis (IPA). List of significant pathways (p-value < 0.05) identified in the set of DEGs according to the

Tender and Tough groups, Table S8: Ingenuity Pathway Analysis (IPA). List of upstream regulators identified in the set of DEGs according to the Tender and Tough group (p-value < 0.05). PAS: Predicted activation ratio. Figure S1: Gene expression distribution of the 25,878 genes annotated in the pig genome (Sscrofa11.1) in fragments per kilobase of transcript per million mapped fragments (FPKMs) normalized values corresponding to the animals with the highest EBV for shear force and tougher meat (High EBV SFF) and the lowest EBV for shear force and tenderer meat (Low EBV SFF). Figure S2: Network of protein–protein interactions predicted with the STRING database. Same color nodes sharing multiple edges are grouped in the same cluster. Figure S3: Gene network #8: Cardiovascular System Development and Function, Cell Cycle, Gene Expression. Genes upregulated and down-regulated in the Tender group are represented in green and red colors, respectively. Figure S4: Gene network #9: Cell Cycle, Cellular Assembly and Organization, Cellular Movement. Genes upregulated and down-regulated in the Tender group are represented in green and red colors, respectively.

Author Contributions: M.Á.F.-B. participated in animal sampling, data analyses, and interpretation and drafting of the manuscript. C.C. participated in animal sampling, RNA isolation and qPCR analyses. Y.N. participated in RNA isolation and qPCR analyses. L.S. and C.R. participated in experimental design, animal sampling. F.S.-E. and G.M. provided and controlled the animal material and participated in animal sampling. J.M.G.-C. participated in animal sampling, supervision of the experiments and drafting of the manuscript. M.M. participated in animal sampling, supervision of data analyses and drafting the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by CON15-078, CON17-125 (INIA-SRC) and IDI-20171141 (CDTI) grants. Miguel Ángel Fernández-Barroso was funded by a FPI Ph.D. grant from the INIA institution.

Acknowledgments: We want to thank to Sánchez Romero Carvajal (SRC) enterprise for the technical support, especially to its manager Jose María Pariente also Fernando Gómez-Carballar. We are gratefully acknowledged to our colleagues, Fabián García and Rita Benitez (INIA) for their technical assistance.

Conflicts of Interest: The authors Fernando Sánchez-Esquiliche and Gema Matos are employees of the enterprise "Sánchez Romero Carvajal" and Miguel Ángel Fernández-Barroso, Carmen Caraballo, Luis Silió, Carmen Rodríguez, Yolanda Nuñez, Juan María García-Casco and María Muñoz are employees of the "Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria—INIA", however we declare no conflicts of interest with regard to the writing of this manuscript.

References

1. Maltin, C.; Balcerzak, D.; Tilley, R.; Delday, M. Determinants of Meat Quality: Tenderness. *Proc. Nutr. Soc.* **2003**, *62*, 337–347. [CrossRef] [PubMed]
2. Kim, J.M.; Choi, B.D.; Kim, B.C.; Park, S.S.; Hong, K.C. Associations of the Variation in the Porcine Myogenin Gene with Muscle Fibre Characteristics, Lean Meat Production and Meat Quality Traits. *J. Anim. Breed. Genet.* **2009**, *126*, 134–141. [CrossRef] [PubMed]
3. Čandek-Potokar, M.; Žlender, B.; Lefaucheur, L.; Bonneau, M. Effects of Age And/or Weight at Slaughter on Longissimus Dorsi Muscle: Biochemical Traits and Sensory Quality in Pigs. *Meat Sci.* **1998**, *48*, 287–300. [CrossRef]
4. Lonergan, S.M.; Huff-Lonergan, E.; Rowe, L.J.; Kuhlers, D.L.; Jungst, S.B. Selection for Lean Growth Efficiency in Duroc Pigs Influences Pork Quality. *J. Anim. Sci.* **2001**, *79*, 2075–2085. [CrossRef]
5. Rosenvold, K.; Andersen, H.J. Factors of Significance for Pork Quality—A Review. *Meat Sci.* **2003**, *64*, 219–237. [CrossRef]
6. Suzuki, K.; Irie, M.; Kadowaki, H.; Shibata, T.; Kumagai, M.; Nishida, A. Genetic Parameter Estimates of Meat Quality Traits in Duroc Pigs Selected for Average Daily Gain, Longissimus Muscle Area, Backfat Thickness, and Intramuscular Fat Content. *J. Anim. Sci.* **2005**, *83*, 2058–2065. [CrossRef]
7. Miar, Y.; Plastow, G.S.; Moore, S.S.; Manafiazar, G.; Charagu, P.; Kemp, R.A.; van Haandel, B.; Huisman, A.E.; Zhang, C.Y.; Mckay, R.M.; et al. Genetic and Phenotypic Parameters for Carcass and Meat Quality Traits in Commercial Crossbred Pigs. *J. Anim. Sci.* **2014**, *92*, 2869–2884. [CrossRef]
8. Fernández-Barroso, M.Á.; Silió, L.; Rodríguez, C.; Palma-Granados, P.; López, A.; Caraballo, C.; García-Casco, J.M.; Sánchez-Esquiliche, F.; Gómez-Carballar, F.; Muñoz, M. Genetic Parameter Estimation and Gene Association Analyses for Meat Quality Traits in Open-Air Free-Range Iberian Pigs. *J. Anim. Breed. Genet.* **2020**, 1–18. [CrossRef]
9. Lindholm-Perry, A.K.; Rohrer, G.A.; Holl, J.W.; Shackelford, S.D.; Wheeler, T.L.; Koohmaraie, M.; Nonneman, D. Relationships among Calpastatin Single Nucleotide Polymorphisms, Calpastatin Expression and Tenderness in Pork Longissimus. *Anim. Genet.* **2009**, *40*, 713–721. [CrossRef]

10. Gandolfi, G.; Pomponio, L.; Ertbjerg, P.; Karlsson, A.H.; Nanni Costa, L.; Lametsch, R.; Russo, V.; Davoli, R. Investigation on CAST, CAPN1 and CAPN3 Porcine Gene Polymorphisms and Expression in Relation to Post-Mortem Calpain Activity in Muscle and Meat Quality. *Meat Sci.* **2011**, *88*, 694–700. [CrossRef]
11. Honikel, K.O. Reference Methods for the Assessment of Physical Characteristics of Meat. *Meat Sci.* **1998**, *49*, 447–457. [CrossRef]
12. Tejerina, D.; García-Torres, S.; Cava, R. Water-Holding Capacity and Instrumental Texture Properties of M. Longissimus Dorsi and M. Serratus Ventralis from Iberian Pigs as Affected by the Production System. *Livest. Sci.* **2012**, *148*, 46–51. [CrossRef]
13. Damon, M.; Wyszynska-Koko, J.; Vincent, A.; Hérault, F.; Lebret, B. Comparison of Muscle Transcriptome between Pigs with Divergent Meat Quality Phenotypes Identifies Genes Related to Muscle Metabolism and Structure. *PLoS ONE* **2012**, *7*, e33763. [CrossRef] [PubMed]
14. Hamill, R.M.; McBryan, J.; McGee, C.; Mullen, A.M.; Sweeney, T.; Talbot, A.; Cairns, M.T.; Davey, G.C. Functional Analysis of Muscle Gene Expression Profiles Associated with Tenderness and Intramuscular Fat Content in Pork. *Meat Sci.* **2012**, *92*, 440–450. [CrossRef] [PubMed]
15. Horodyska, J.; Wimmers, K.; Reyer, H.; Trakooljul, N.; Mullen, A.M.; Lawlor, P.G.; Hamill, R.M. RNA-Seq of Muscle from Pigs Divergent in Feed Efficiency and Product Quality Identifies Differences in Immune Response, Growth, and Macronutrient and Connective Tissue Metabolism. *BMC Genom.* **2018**, *19*, 1–18. [CrossRef] [PubMed]
16. Rivera-Ferre, M.G.; Aguilera, J.F.; Nieto, R. Muscle Fractional Protein Synthesis Is Higher in Iberian than in Landrace Growing Pigs Fed Adequate or Lysine-Deficient Diets. *J. Nutr.* **2005**, *135*, 469–478. [CrossRef]
17. Lopez-Bote, C.J. Sustained Utilization of the Iberian Pig Breed. *Meat Sci.* **1998**, *49*. [CrossRef]
18. Alves, E.; Óvilo, C.; Rodríguez, M.C.; Silió, L. Mitochondrial DNA Sequence Variation and Phylogenetic Relationships among Iberian Pigs and Other Domestic and Wild Pig Populations. *Anim. Genet.* **2003**, *34*, 319–324. [CrossRef]
19. Fabuel, E.; Barragán, C.; Silió, L.; Rodríguez, M.C.; Toro, M.A. Analysis of Genetic Diversity and Conservation Priorities in Iberian Pigs Based on Microsatellite Markers. *Heredity* **2004**, *93*, 104–113. [CrossRef]
20. Ollivier, L. European Pig Genetic Diversity: A. Minireview. *Animal* **2009**, *3*, 915–924. [CrossRef]
21. Muñoz, M.; Sánchez-Esquiliche, F.; Caraballo, C.; Gómez, F.; Pariente, J.M.; Silió, L.; Rodríguez, C.; García-Casco, J.M. Animal Breeding Scheme Applied to the Quality of Pure Iberian Montanera Pigs. *Arch. Zootec.* **2018**, *67*, 9–11. [CrossRef]
22. Combes, S.; Lepetit, J.; Darche, B.; Lebas, F. Effect of Cooking Temperature and Cooking Time on Warner-Bratzler Tenderness Measurement and Collagen Content in Rabbit Meat. *Meat Sci.* **2004**, *66*, 91–96. [CrossRef]
23. Legarra, A.; Varona, L.; de Maturana, E.L. *TM Threshold Model*; INRA-SAGA: Toulouse, France, 2008.
24. Muñoz, M.; García-Casco, J.M.; Caraballo, C.; Fernández-Barroso, M.Á.; Sánchez-Esquiliche, F.; Gómez, F.; Rodríguez, M.d.C.; Silió, L. Identification of Candidate Genes and Regulatory Factors Underlying Intramuscular Fat Content Through Longissimus Dorsi Transcriptome Analyses in Heavy Iberian Pigs. *Front. Genet.* **2018**, *9*, 1–16. [CrossRef] [PubMed]
25. Trapnell, C.; Pachter, L.; Salzberg, S.L. TopHat: Discovering Splice Junctions with RNA-Seq. *Bioinformatics* **2009**, *25*, 1105–1111. [CrossRef] [PubMed]
26. Trapnell, C.; Roberts, A.; Goff, L.; Pertea, G.; Kim, D.; Kelley, D.R.; Pimentel, H.; Salzberg, S.L.; Rinn, J.L.; Pachter, L. Differential Gene and Transcript Expression Analysis of RNA-Seq Experiments with TopHat and Cufflinks. *Nat. Protoc.* **2012**, *7*, 562–578. [CrossRef]
27. Storey, J.D.; Tibshirani, R. Statistical Significance for Genomewide Studies. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9440–9445. [CrossRef]
28. Szklarczyk, D.; Morris, J.H.; Cook, H.; Kuhn, M.; Wyder, S.; Simonovic, M.; Santos, A.; Doncheva, N.T.; Roth, A.; Bork, P.; et al. The STRING Database in 2017: Quality-Controlled Protein-Protein Association Networks, Made Broadly Accessible. *Nucleic Acids Res.* **2017**, *45*, D362–D368. [CrossRef]
29. Krämer, A.; Green, J.; Pollard, J.; Tugendreich, S. Causal Analysis Approaches in Ingenuity Pathway Analysis. *Bioinformatics* **2014**, *30*, 523–530. [CrossRef]
30. Vandesompele, J.; De Preter, K.; Pattyn, F.; Poppe, B.; Van Roy, N.; De Paepe, A.; Speleman, F. Accurate Normalization of Real-Time Quantitative RT-PCR Data by Geometric Averaging of Multiple Internal Control Genes. *Genome Biol.* **2002**, *3*, research0034-1. [CrossRef]

31. Miron, M.; Woody, O.Z.; Marcil, A.; Murie, C.; Sladek, R.; Nadon, R. A Methodology for Global Validation of Microarray Experiments. *BMC Bioinform.* **2006**, *7*. [CrossRef]
32. Zappaterra, M.; Gioiosa, S.; Chillemi, G.; Zambonelli, P.; Davoli, R. Muscle Transcriptome Analysis Identifies Genes Involved in Ciliogenesis and the Molecular Cascade Associated with Intramuscular Fat Content in Large White Heavy Pigs. *PLoS ONE* **2020**, *15*, e0233372. [CrossRef] [PubMed]
33. Roberts, A.; Pimentel, H.; Trapnell, C.; Pachter, L. Identification of Novel Transcripts in Annotated Genomes Using RNA-Seq. *Bioinformatics* **2011**, *27*, 2325–2329. [CrossRef] [PubMed]
34. Kemp, C.M.; Sensky, P.L.; Bardsley, R.G.; Buttery, P.J.; Parr, T. Tenderness—An Enzymatic View. *Meat Sci.* **2010**, *84*, 248–256. [CrossRef] [PubMed]
35. Hopkins, D.; Taylor. Post-Mortem Muscle Proteolysis and Meat Tenderness. In *Muscle Development of Livestock Animals: Physiology, Genetics, and Meat Quality*; Centre for Agriculture and Bioscience International: Wallingford, UK, 2004; pp. 363–388.
36. Li, S.; Xu, X.; Zhou, G. The Roles of the Actin-Myosin Interaction and Proteolysis in Tenderization during the Aging of Chicken Muscle. *Poult. Sci.* **2012**, *91*, 150–160. [CrossRef] [PubMed]
37. D'Andrea, M.; Dal Monego, S.; Pallavicini, A.; Modonut, M.; Dreos, R.; Stefanon, B.; Pilla, F. Muscle Transcriptome Profiling in Divergent Phenotype Swine Breeds during Growth Using Microarray and RT-PCR Tools. *Anim. Genet.* **2011**, *42*, 501–509. [CrossRef] [PubMed]
38. Piórkowska, K.; Żukowski, K.; Ropka-Molik, K.; Tyra, M. Detection of Genetic Variants between Different Polish Landrace and Puławska Pigs by Means of RNA-Seq Analysis. *Anim. Genet.* **2018**, *49*, 215–225. [CrossRef] [PubMed]
39. Casiró, S.; Velez-Irizarry, D.; Ernst, C.W.; Raney, N.E.; Bates, R.O.; Charles, M.G.; Steibel, J.P. Genome-Wide Association Study in an F2 Duroc X Pietrain Resource Population for Economically Important Meat Quality and Carcass Traits. *J. Anim. Sci.* **2017**, *95*, 545–558. [CrossRef]
40. Gad, A.K.B.; Nehru, V.; Ruusala, A.; Aspenström, P. RhoD Regulates Cytoskeletal Dynamics via the Actin Nucleation-Promoting Factor WASp Homologue Associated with Actin Golgi Membranes and Microtubules. *Mol. Biol. Cell* **2012**, *23*, 4807–4819. [CrossRef]
41. Toydemir, R.M.; Chen, H.; Proud, V.K.; Martin, R.; Van Bokhoven, H.; Hamel, B.C.J.; Tuerlings, J.H.; Stratakis, C.A.; Jorde, L.B.; Bamshad, M.J. Trismus-Pseudocamptodactyly Syndrome Is Caused by Recurrent Mutation of MYH8. *Am. J. Med. Genet. Part A* **2006**, *140*, 2387–2393. [CrossRef]
42. Karsch-Mizrachi, I.; Feghali, R.; Shows, T.B.; Leinwand, L.A. Generation of a Full-Length Human Perinatal Myosin Heavy-Chain-Encoding cDNA. *Gene* **1990**, *89*, 289–294. [CrossRef]
43. Liu, S.; Han, W.; Jiang, S.; Zhao, C.; Wu, C. Integrative Transcriptomics and Proteomics Analysis of Longissimus Dorsi Muscles of Canadian Double-Muscled Large White Pigs. *Gene* **2016**, *577*, 14–23. [CrossRef] [PubMed]
44. Sparrow, J.C.; Nowak, K.J.; Durling, H.J.; Beggs, A.H.; Wallgren-Pettersson, C.; Romero, N.; Nonaka, I.; Laing, N.G. Muscle Disease Caused by Mutations in the Skeletal Muscle Alpha-Actin Gene (ACTA1). *Neuromusc. Disord.* **2003**, 519–531. [CrossRef]
45. Nowak, K.J.; Wattanasirichaigoon, D.; Goebel, H.H.; Wilce, M.; Pelin, K.; Donner, K.; Jacob, R.L.; Hübner, C.; Oexle, K.; Anderson, J.R.; et al. Mutations in the Skeletal Muscle Alpha-Actin Gene in Patients with Actin Myopathy and Nemaline Myopathy. *Nat. Genet.* **1999**, *23*, 208–212. [CrossRef] [PubMed]
46. Yang, H.; Xu, X.; Li, M.; Ming, H.; Jiang, J. Integrative Analysis of Transcriptomics and Proteomics of Skeletal Muscles of the Chinese Indigenous Shaziling Pig Compared with the Yorkshire Breed. *BMC Genet.* **2016**, *17*, 1–13. [CrossRef] [PubMed]
47. Ponsuksili, S.; Siengdee, P.; Du, Y.; Trakooljul, N.; Murani, E.; Schwerin, M.; Wimmers, K. Identification of Common Regulators of Genes in Co-Expression Networks Affecting Muscle and Meat Properties. *PLoS ONE* **2015**, *10*, e0123678. [CrossRef]
48. Jerez-Timaure, N.; Gallo, C.; Ramírez-Reveco, A.; Greif, G.; Strobel, P.; Pedro, A.V.F.; Morera, F.J. Early Differential Gene Expression in Beef Longissimus Thoracis Muscles from Carcasses with Normal (<5.8) and High (>5.9) Ultimate pH. *Meat Sci.* **2019**, *153*, 117–125. [CrossRef]
49. Sentandreu, M.A.; Coulis, G.; Ouali, A. Role of Muscle Endopeptidases and Their Inhibitors in Meat Tenderness. *Trends Food Sci. Technol.* **2002**, *13*, 400–421. [CrossRef]
50. Goll, D.E.; Thompson, V.F.; Li, H.; Wei, W.; Cong, J. The Calpain System. *Physiol. Rev.* **2003**, *1990*, 731–801. [CrossRef]

51. Koohmaraie, M.; Geesink, G.H. Contribution of Postmortem Muscle Biochemistry to the Delivery of Consistent Meat Quality with Particular Focus on the Calpain System. *Meat Sci.* **2006**, *74*, 34–43. [CrossRef]
52. Van Laack, R.L.J.M.; Stevens, S.G.; Stalder, K.J. The Influence of Ultimate pH and Intramuscular Fat Content on Pork Tenderness and Tenderization. *J. Anim. Sci.* **2001**, *79*, 392–397. [CrossRef]
53. Wu, C.; Dedhar, S. Integrin-Linked Kinase (ILK) and Its Interactors: A New Paradigm for the Coupling of Extracellular Matrix to Actin Cytoskeleton and Signaling Complexes. *J. Cell Biol.* **2001**, 505–510. [CrossRef] [PubMed]
54. Giancotti, F.G.; Ruoslahti, E. Integrin Signaling. *Science* **1999**, 1028–1032. [CrossRef] [PubMed]
55. Ouali, A.; Herrera-Mendez, C.H.; Coulis, G.; Becila, S.; Boudjellal, A.; Aubry, L.; Sentandreu, M.A. Revisiting the Conversion of Muscle into Meat and the Underlying Mechanisms. *Meat Sci.* **2006**, *74*, 44–58. [CrossRef] [PubMed]
56. Torrado, M.; Nespereira, B.; López, E.; Centeno, A.; Castro-Beiras, A.; Mikhailov, A.T. ANKRD1 Specifically Binds CASQ2 in Heart Extracts and Both Proteins Are Co-Enriched in Piglet Cardiac Purkinje Cells. *J. Mol. Cell. Cardiol.* **2005**, *38*, 353–365. [CrossRef] [PubMed]
57. Ovilo, C.; Benítez, R.; Fernández, A.; Núñez, Y.; Ayuso, M.; Fernández, A.I.; Rodríguez, C.; Isabel, B.; Rey, A.I.; López-Bote, C.; et al. Longissimus Dorsi Transcriptome Analysis of Purebred and Crossbred Iberian Pigs Differing in Muscle Characteristics. *BMC Genom.* **2014**, *15*, 413. [CrossRef]
58. Li, X.J.; Zhou, J.; Liu, L.Q.; Qian, K.; Wang, C.L. Identification of Genes in Longissimus Dorsi Muscle Differentially Expressed between Wannanhua and Yorkshire Pigs Using RNA-Sequencing. *Anim. Genet.* **2016**, *47*, 324–333. [CrossRef]
59. Ponsuksili, S.; Murani, E.; Phatsara, C.; Schwerin, M.; Schellander, K.; Wimmers, K. Porcine Muscle Sensory Attributes Associate with Major Changes in Gene Networks Involving CAPZB, ANKRD1, and CTBP2. *Funct. Integr. Genom.* **2009**, *9*, 455–471. [CrossRef]
60. Velez-Irizarry, D.; Casiro, S.; Daza, K.R.; Bates, R.O.; Raney, N.E.; Steibel, J.P.; Ernst, C.W. Genetic Control of Longissimus Dorsi Muscle Gene Expression Variation and Joint Analysis with Phenotypic Quantitative Trait Loci in Pigs 06 Biological Sciences 0604 Genetics. *BMC Genom.* **2019**, *20*, 1–19. [CrossRef]
61. Zhu, J.; Shi, X.E.; Lu, H.; Xia, B.; Li, Y.; Li, X.; Zhang, Q.; Yang, G. RNA-Seq Transcriptome Analysis of Extensor Digitorum Longus and Soleus Muscles in Large White Pigs. *Mol. Genet. Genom.* **2016**, *291*, 687–701. [CrossRef]
62. Kambadur, R.; Sharma, M.; Smith, T.P.L.; Bass, J.J. Mutations in Myostatin (GDF8) in Double-Muscled Belgian Blue and Piedmontese Cattle. *Genome Res.* **1997**, *7*, 910–916. [CrossRef]
63. Clop, A.; Marcq, F.; Takeda, H.; Pirottin, D.; Tordoir, X.; Bibé, B.; Bouix, J.; Caiment, F.; Elsen, J.M.; Eychenne, F.; et al. A Mutation Creating a Potential Illegitimate microRNA Target Site in the Myostatin Gene Affects Muscularity in Sheep. *Nat. Genet.* **2006**, *38*, 813–818. [CrossRef] [PubMed]
64. Qian, L.; Tang, M.; Yang, J.; Wang, Q.; Cai, C.; Jiang, S.; Li, H.; Jiang, K.; Gao, P.; Ma, D.; et al. Targeted Mutations in Myostatin by Zinc-Finger Nucleases Result in Double-Muscled Phenotype in Meishan Pigs. *Sci. Rep.* **2015**, *5*. [CrossRef] [PubMed]
65. Deng, B.; Zhang, F.; Wen, J.; Ye, S.; Wang, L.; Yang, Y.; Gong, P.; Jiang, S. The Function of Myostatin in the Regulation of Fat Mass in Mammals. *Nutr. Metab.* **2017**. [CrossRef]
66. Haenggi, T.; Fritschy, J.M. Role of Dystrophin and Utrophin for Assembly and Function of the Dystrophin Glycoprotein Complex in Non-Muscle Tissue. *Cell. Mol. Life Sci.* **2006**, 1614–1631. [CrossRef] [PubMed]
67. Fröhlich, T.; Kemter, E.; Flenkenthaler, F.; Klymiuk, N.; Otte, K.A.; Blutke, A.; Krause, S.; Walter, M.C.; Wanke, R.; Wolf, E.; et al. Progressive Muscle Proteome Changes in a Clinically Relevant Pig Model of Duchenne Muscular Dystrophy. *Sci. Rep.* **2016**, *6*. [CrossRef]
68. Ferrero, G.O.; Velazquez, F.N.; Caputto, B.L. The Kinase c-Src and the Phosphatase TC45 Coordinately Regulate c-Fos Tyrosine Phosphorylation and c-Fos Phospholipid Synthesis Activation Capacity. *Oncogene* **2012**, *31*, 3381–3391. [CrossRef]
69. Reiner, G.; Heinricy, L.; Müller, E.; Geldermann, H.; Dzapo, V. Indications of Associations of the Porcine FOS Proto-Oncogene with Skeletal Muscle Fibre Traits. *Anim. Genet.* **2002**, *33*, 49–55. [CrossRef]
70. Rajan, S.; Dang, H.C.P.; Djambazian, H.; Zuzan, H.; Fedyshyn, Y.; Ketela, T.; Moffat, J.; Hudson, T.J.; Sladek, R. Analysis of Early C2C12 Myogenesis Identifies Stably and Differentially Expressed Transcriptional Regulators Whose Knock-down Inhibits Myoblast Differentiation. *Physiol. Genom.* **2012**, *44*, 183–197. [CrossRef]

71. Ayuso, M.; Fernández, A.; Núñez, Y.; Benitez, R.; Isabel, B.; Barragán, C.; Fernández, A.I.; Rey, A.I.; Medrano, J.F.; Cánovas, Á.; et al. Comparative Analysis of Muscle Transcriptome between Pig Genotypes Identifies Genes and Regulatory Mechanisms Associated to Growth, Fatness and Metabolism. *PLoS ONE* **2015**, *10*, e0145162. [CrossRef]
72. García-Contreras, C.; Madsen, O.; Groenen, M.A.M.; López-García, A.; Vázquez-Gómez, M.; Astiz, S.; Núñez, Y.; Benítez, R.; Fernández, A.; Isabel, B.; et al. Impact of Genotype, Body Weight and Sex on the Prenatal Muscle Transcriptome of Iberian Pigs. *PLoS ONE* **2020**, *15*, e0227861. [CrossRef]
73. Migita, T.; Narita, T.; Asaka, R.; Miyagi, E.; Nagano, H.; Nomura, K.; Matsuura, M.; Satoh, Y.; Okumura, S.; Nakagawa, K.; et al. Role of Insulin-like Growth Factor Binding Protein 2 in Lung Adenocarcinoma: IGF-Independent Antiapoptotic Effect via Caspase-3. *Am. J. Pathol.* **2010**, *176*, 1756–1766. [CrossRef] [PubMed]
74. Hakuno, F.; Takahashi, S.-I. IGF1 Receptor Signaling Pathways. *J. Mol. Endocrinol.* **2018**, *61*, T69–T86. [CrossRef] [PubMed]
75. Wood, J.D. Consequences for Meat Quality of Reducing Carcass Fatness. In *Reducing Fat in Meat Animals*; Elsevier: Amsterdam, The Netherlands, 1990; pp. 344–397.
76. Lee, J.H.; Song, K.D.; Lee, H.K.; Cho, K.H.; Park, H.C.; Park, K.D. Genetic Parameters of Reproductive and Meat Quality Traits in Korean Berkshire Pigs. *Asian-Australas. J. Anim. Sci.* **2015**, *28*, 1388–1393. [CrossRef]
77. Sellier, P. *Genetics of Meat and Carcass Traits. Genetics of the Pig*; Centre for Agriculture and Bioscience International: Wallingford, UK, 1998; pp. 463–510.
78. Laville, E.; Sayd, T.; Terlouw, C.; Chambon, C.; Damon, M.; Larzul, C.; Leroy, P.; Glénisson, J.; Chérel, P. Comparison of Sarcoplasmic Proteomes between Two Groups of Pig Muscles Selected for Shear Force of Cooked Meat. *J. Agric. Food Chem.* **2007**, *55*, 5834–5841. [CrossRef] [PubMed]
79. Óvilo, C.; Oliver, A.; Noguera, J.L.; Clop, A.; Barragán, C.; Varona, L.; Rodríguez, C.; Toro, M.; Sánchez, A.; Pérez-Enciso, M.; et al. Test for Positional Candidate Genes for Body Composition on Pig Chromosome 6. *Genet. Sel. Evol.* **2002**, *34*, 465–479. [CrossRef]
80. Li, X.; Kim, S.W.; Choi, J.S.; Lee, Y.M.; Lee, C.K.; Choi, B.H.; Kim, T.H.; Choi, Y.I.; Kim, J.J.; Kim, K.S. Investigation of Porcine FABP3 and LEPR Gene Polymorphisms and mRNA Expression for Variation in Intramuscular Fat Content. *Mol. Biol. Rep.* **2010**, *37*, 3931–3939. [CrossRef] [PubMed]
81. Chen, J.N.; Jiang, Y.Z.; Cen, W.M.; Xing, S.H.; Zhu, L.; Tang, G.Q.; Li, M.Z.; Jiang, A.A.; Lou, P.E.; Wen, A.X.; et al. Distribution of H-FABP and ACSL4 Gene Polymorphisms and Their Associations with Intramuscular Fat Content and Backfat Thickness in Different Pig Populations. *Genet. Mol. Res.* **2014**, *13*, 6759–6772. [CrossRef]
82. Handschin, C.; Spiegelman, B.M. Peroxisome Proliferator-Activated Receptor γ Coactivator 1 Coactivators, Energy Homeostasis, and Metabolism. *Endocr. Rev.* **2006**, *27*, 728–735. [CrossRef]
83. Lin, J.; Wu, H.; Tarr, P.T.; Zhang, C.Y.; Wu, Z.; Boss, O.; Michael, L.F.; Puigserver, P.; Isotani, E.; Olson, E.N.; et al. Transcriptional Co-Activator PGC-1α Drives the Formation of Slow-Twitch Muscle Fibres. *Nature* **2002**, *418*, 797–801. [CrossRef]
84. Erkens, T.; De Smet, S.; Van Den Maagdenberg, K.; Stinckens, A.; Buys, N.; Van Zeveren, A.; Peelman, L.J. Association Analysis of PPARGC1A Mutations with Meat Quality Parameters in a Commercial Hybrid Pig Population. *Czech J. Anim. Sci.* **2010**, *55*, 200–208. [CrossRef]
85. Chambon, P. The Nuclear Receptor Superfamily: A Personal Retrospect on the First Two Decades. *Mol. Endocrinol.* **2005**, 1418–1428. [CrossRef] [PubMed]
86. Hocquette, J.F.; Gondret, F.; Baza, E.; Mdale, F.; Jurie, C.; Pethick, D.W. Intramuscular Fat Content in Meat-Producing Animals: Development, Genetic and Nutritional Control, and Identification of Putative Markers. *Animal* **2010**, *4*, 303–319. [CrossRef] [PubMed]
87. Weston, A.R.; Rogers, R.W.; Althen, T.G. REVIEW: The Role of Collagen in Meat Tenderness. *Prof. Anim. Sci.* **2002**, *18*, 107–111. [CrossRef]
88. Purslow, P.P. Intramuscular Connective Tissue and Its Role in Meat Quality. *Meat Sci.* **2005**, *70*, 435–447. [CrossRef] [PubMed]
89. Torrescano, G.; Sánchez-Escalante, A.; Giménez, B.; Roncalés, P.; Beltrán, J.A. Shear Values of Raw Samples of 14 Bovine Muscles and Their Relation to Muscle Collagen Characteristics. *Meat Sci.* **2003**, *64*, 85–91. [CrossRef]

90. Ngapo, T.M.; Berge, P.; Culioli, J.; Dransfield, E.; De Smet, S.; Claeys, E. Perimysial Collagen Crosslinking and Meat Tenderness in Belgian Blue Double-Muscled Cattle. *Meat Sci.* **2002**, *61*, 91–102. [CrossRef]
91. Dransfield, E.; Martin, J.F.; Bauchart, D.; Abouelkaram, S.; Lepetit, J.; Culioli, J.; Jurie, C.; Picard, B. Meat Quality and Composition of Three Muscles from French Cull Cows and Young Bulls. *Anim. Sci.* **2003**, *76*, 387–399. [CrossRef]
92. Lepetit, J. A Theoretical Approach of the Relationships between Collagen Content, Collagen Cross-Links and Meat Tenderness. *Meat Sci.* **2007**, *76*, 147–159. [CrossRef]
93. Schuppan, D.; Cantaluppi, M.C.; Becker, J.; Veit, A.; Bunte, T.; Troyer, D.; Schuppan, F.; Schmid, M.; Ackermann, R.; Hahn, E.G. Undulin, an Extracellular Matrix Glycoprotein Associated with Collagen Fibrils. *J. Biol. Chem.* **1990**, *265*, 8823–8832.
94. Xu, J.; Wang, C.; Jin, E.; Gu, Y.; Li, S.; Li, Q. Identification of Differentially Expressed Genes in Longissimus Dorsi Muscle between Wei and Yorkshire Pigs Using RNA Sequencing. *Genes Genom.* **2018**, *40*, 413–421. [CrossRef]
95. Hishida, T.; Nishizuka, M.; Osada, S.; Imagawa, M. The Role of C/EBPδ in the Early Stages of Adipogenesis. *Biochimie* **2009**, *91*, 654–657. [CrossRef] [PubMed]
96. Pena, R.N.; Noguera, J.L.; García-Santana, M.J.; González, E.; Tejeda, J.F.; Ros-Freixedes, R.; Ibáñez-Escriche, N. Five Genomic Regions Have a Major Impact on Fat Composition in Iberian Pigs. *Sci. Rep.* **2019**, *9*. [CrossRef] [PubMed]
97. Figeac, N.; Mohamed, A.D.; Sun, C.; Schönfelder, M.; Matallanas, D.; Garcia-Munoz, A.; Missiaglia, E.; Collie-Duguid, E.; De Mello, V.; Pobbati, A.V.; et al. VGLL3 Operates via TEAD1, TEAD3 and TEAD4 to Influence Myogenesis in Skeletal Muscle. *J. Cell Sci.* **2019**, *132*, 1–15. [CrossRef] [PubMed]
98. Zimmerman, A.W.; Veerkamp, J.H. New Insights into the Structure and Function of Fatty Acid-Binding Proteins. *Cell. Mol. Life Sci.* **2002**, *59*, 1096–1116. [CrossRef] [PubMed]
99. Sanchez, M.P.; Iannuccelli, N.; Basso, B.; Bidanel, J.P.; Billon, Y.; Gandemer, G.; Gilbert, H.; Larzul, C.; Legault, C.; Riquet, J.; et al. Identification of QTL with Effects on Intramuscular Fat Content Fatty Acid Composition in a Duroc × Large White Cross. *BMC Genet.* **2007**, *8*. [CrossRef] [PubMed]
100. Ayuso, M.; Fernández, A.; Núñez, Y.; Benítez, R.; Isabel, B.; Fernández, A.I.; Rey, A.I.; González-Bulnes, A.; Medrano, J.F.; Cánovas, Á.; et al. Developmental Stage, Muscle and Genetic Type Modify Muscle Transcriptome in Pigs: Effects on Gene Expression and Regulatory Factors Involved in Growth and Metabolism. *PLoS ONE* **2016**, *11*, e0167858. [CrossRef]

CAPÍTULO IV

Understanding the role of myoglobin content in Iberian pigs fattened in an extensive system through analysis of the transcriptome profile.

Fernández-Barroso, M.A., Muñoz, M., Núñez, Y., Ramírez-Hidalgo, L., Matos G., García-Casco J.M. 2021.

Understanding the role of myoglobin content in Iberian pigs fattened in an extensive system through analysis of the transcriptome profile

Miguel Ángel Fernández-Barroso[1,2*], María Muñoz[2], Yolanda Núñez[2], Luisa Ramirez-Hidalgo[3], Gema Matos[3] and Juan María García-Casco[1,2]

[1] Centro Nacional de I+D del Cerdo Ibérico, INIA, 06300 Zafra, Spain; garcia.juan@inia.es (J.M.G.-C.)

[2]Departamento de Mejora Genética Animal, INIA, 28040 Madrid, Spain; nunez.yolanda@inia.es (Y.N.); mariamm@inia.es (M.M.)

[3]Sánchez Romero Carvajal—Jabugo, SRC, 21290 Huelva, Spain; luisa.ramirez@osborne.es (L.R-H.); gema.matos@osborne.es (G.M.)

*Correspondence: Miguel Ángel Fernández-Barroso, Centro de I+D en Cerdo Ibérico, INIA, Zafra, Spain. Departamento de Mejora Genética Animal, INIA, Madrid, Spain.

Summary

Meat colour is the first perceived sensory feature and one of the most important quality traits. Myoglobin is the main pigment in meat, giving its characteristic cherry-red colour which is highly appreciated by consumers. In the current study, we used the RNA-seq technique to characterise the longissimus dorsi muscle transcriptome in two groups of Iberian pigs with divergent breeding values for myoglobin content. As a result, we identified 57 differentially expressed genes and transcripts (DEG). Moreover, we have validated the RNA-seq expression of a set of genes by quantitative PCR (qPCR). Functional analyses revealed an enrichment of DEGs in biological process related to oxidation (*HBA1*), lipid metabolism (*ECH1, PLA2G10, PLD2*), inflammation (*CHST1, CD209, PLA2G10*), and immune system (*CD209, MX2, LGALS3, LGALS9*). The upstream analysis showed a total of five transcription regulatory factors and eight master regulators that could regulate the expression of some DEGs, highlighting SPI1 and MAPK1, since they regulate the expression of DEGs involved in immune defence and inflammatory processes. Iberian pigs with high myoglobin content also showed higher expression of the *HBA1* gene and it has been described that both molecules have a protective effect against oxidative and inflammatory processes. Therefore, the *HBA1* gene is a very promising candidate gene to harbour polymorphisms underlying the

myoglobin content, whereby, further studies should be carried out for its potential use in an Iberian pig selection program.

Keywords: myoglobin content, Iberian pig, RNA-seq, transcriptome and functional analysis.

1. Introduction

Meat colour is considered one of the most important meat quality traits and the first attribute that is sensory perceived, influencing the purchase decision of consumers (Mancini and Hunt, 2005). A bright cherry-red colour is normally used as a healthy indicator in fresh meat, while PSE (pale, soft, exudative) or DFD (dark, firm, dry) meats do not meet the consumer preferences (Yu *et al.*, 2017).

Myoglobin (MB) is the main heme sarcoplasmic protein responsible for oxygen transport and the principal pigment related to meat red colour (Suman *et al.*, 2013). Besides, MB is involved in the oxidative phosphorylation (Wittenberg and Wittenberg, 2003), as well as oxygen binder and delivery to the mitochondria in the skeletal muscle (Suman *et al.*, 2013). Structurally, MB is a monomeric heme protein composed of a heme prosthetic group and a globin protein (Suman *et al.*, 2013). The heme group, which characterizes MB as a pigment, absorbs visible light through its double bonds and contains an iron atom that can be present in reduced (ferrous/Fe^{2+}) or oxidised (ferric/Fe^{3+}) form. The heme group can reversibly bind to ligands such as oxygen, carbon monoxide or nitric oxide. Therefore, there are four redox states of MB: deoxymyoglobin (reduced, DMB), oxymyoglobin (oxygenated, OMB), metmyoglobin (oxidised, MetMB) and carboxymyoglobin (COMB) (Mancini and Hunt, 2005). OMB gives bright cherry-red colour to the meat, critical for consumer acceptance. DMB provides purplish-red colour and MetMB produces a brown colour on meat. These four redox forms of MB can be identified spectrophotometrically and their absorbance spectra range between 500 and 600 nm, with 525 nm being the point at which the absorption spectral curves of the four forms converge (Tang *et al.*, 2004).

The myoglobin content is influenced by different factors such as the species, breed, metabolic profile of the muscle (oxidative or glycolytic), age and production system (extensive or intensive) (Olsson and Pickova, 2005, Ventanas *et al.*, 2005, Yu *et*

al., 2017). In fact, several authors have reported that the meat of pigs managed in open-air extensive systems has a higher myoglobin content (Ventanas *et al.*, 2005), being more reddish and less pale (Pugliese *et al.*, 2005). Moreover, from a genetic point of view, the heritability of this trait estimated both in lean and autochthonous pig breeds showed moderate values. Newcom *et al.* (2004) estimated an average heritability of 0.27 for MB in seven different breeds, while Fernández-Barroso *et al.* (2020a) estimated a value of 0.15 in the Iberian breed. Kim *et al.* (2010) found that MB content was phenotypically correlated (r = 0.45) with a* colour trait (measured by colorimeter) in crossbred pigs between Korean native black pig and Landrace, and Newcom *et al.* (2004) and Fernández-Barroso *et al.* (2020a) estimated a MB-a* positive genetic correlation of 0.23 and 0.94, respectively. Furthermore, some polymorphisms have been identified in candidate genes such as *CASP9* and *PRKAG3* (Fernández-Barroso *et al.*, 2020a, Lindahl *et al.*, 2004) that affect MB content.

Iberian pig breed is characterized by having high quality meat and by their dry-cured products greatly appreciated and with an elevated economic value in the market. The quality of its products is favoured by its particular characteristics, such as the voracious appetite, the high adipogenic potential and protein turnover ratio and the low deposition of lean tissue (Rivera-Ferre *et al.*, 2005) which in turn are determined by its unique traditional open-air production system (Lopez-Bote, 1998) and its genetic features (Alves *et al.*, 2003, Fabuel *et al.*, 2004, Ollivier *et al.*, 2009).

The measurement of MB content is not a straightforward technique (Hornsey, 1956) and it might be advisable to use molecular information to include MB as a selection goal in a breeding program. The sequencing of the whole transcriptome of divergent individuals for a particular trait allows the identification of candidate genes for these traits and, at the same time, a better understanding of the gene networks and biological pathways underlying the analysed trait. The analyses of changes in the transcriptome between divergent individuals for a particular trait such as intramuscular fat, tenderness or feed efficiency through RNA-seq have been carried out in different studies (Muñoz *et al.*, 2018, Vigors *et al*. 2019, Fernández-Barroso *et al*., 2020b, Zapaterra *et al*. 2020). However, to our knowledge, there is no any published study in which the transcriptome of divergent pigs for MB content has been analysed.

In the current study we sequenced the transcriptome of longissimus dorsi (LD) muscle in divergent Iberian pigs for MB content. Hence, the aims of this study were: (a) to identify and quantify differentially expressed genes (DEGs) between divergent groups, (b) carry out *in-silico* functional analyses for a better comprehension of the biological pathways that could be involved in the differences in MB content, (c) identify the transcription regulatory factors influencing the observed gene expression profiles.

2. Materials and Methods

2.1. Animal material and phenotypic data

Animal handling was carried out according to the regulations of the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63/EU about the protection of animals used in research. Protocols were assessed and approved by the INIA Committee of Ethics in Animal Research, which is the named Institutional Animal Care and Use Committee (IACUC) for the INIA.

The animal material used in the present study was obtained from castrated males belonging to a closed commercial population of Iberian pigs. The animals were fed under a restricted feeding regime until they reached 100 kg of body weight and were subsequently fattened in an open-air free-range system until slaughter, with an approximate age of 17 months and 165 kg of final body weight.

After slaughter, LD samples were removed from the carcass of 828 animals and a central muscle section of approximately 80 g was separated of each loin for MB determination. The muscle portions were vacuum packed in nylon/polyethylene bags, and then the samples were introduced in liquid N_2 for approximately 20 seconds, before storing at −20 ºC until determination of MB content. After that, the samples were thawed and MB was measured as mg myoglobin/g muscle as described in Fernández-Barroso *et al.*, 2020a following Horsney (1956) with Alberti *et al.* (2005) modifications. The MB mean was 1.77 mg/g (SD = 0.31).

The following mixed model was used to estimate breeding values (EBVs) for MB content:

$$\mathbf{y = Xb + Za + Wsm + e}$$

where **y** is the vector of MB values corresponding to each animal, **b** represents the vectors of systematic effects, in which the intramuscular fat percentage (IMF), the age and the average weight of the two loins for each individual were fitted as covariates, **a** is the vector of the additive genetic effects (EBVs) distributed as N (0, $\boldsymbol{A\sigma^2_u}$), where A is the numerator of kinship matrix that allows for the adjustment of the data taking into account the pedigree information, **sm** is the vector of the environmental random effects caused by the combined fattening-slaughter batches (24 levels) and **e,** the vector including the residual effects. **X, Z and W** are the incidence matrices. EBVs were estimated using the PEST 4.1 (Groeneveld, Kovak, & Wang, 1999) and VCE-6 programs (Groeneveld, Kovak, & Mielenz, 2010).

A total of 12 pigs with the most extreme EBVs for MB were selected, six per each group, avoiding full and half siblings. In the current study, the most extreme EBVs animals belonged to the same season; therefore, some possible environmental effects associated with the annual differences were reduced. The mean phenotypic values of MB content were 2.48 mg/g (SD = 0.07) for the six individuals that showed the highest EBVs (High MB group) and 1.39 mg/g (SD = 0.15) for the six with the lowest EBVs (Low MB group); the corresponding EBVs averages were 0.18 (SD = 0.02) and -0.20 (SD = 0.04), respectively.

2.2. Transcriptomic analyses

2.2.1. RNA extraction, library preparation and sequencing

The loin samples collected after slaughter were introduced in cryogenic tubes, frozen in liquid nitrogen and stored at −80 °C until analysis. The RiboPure™ High-Quality RNA Purification kit (Ambion, Austin, TX, USA) was used to extract total RNA, following the manufacturer's recommendations. NanoDrop equipment (NanoDrop Technologies, Wilmington, DE, USA) was used to quantify the RNA. Agilent 2100 Bioanalyzer device (Agilent Technologies, Santa Clara, CA, USA) was used to measure RNA integrity (RNA Integrity Number = RIN); the RIN values obtained for all the samples were higher than 8.

NEBNext® Ultra™ RNA Library Prep Kit (Illumina, San Diego, CA, USA) was used to build the paired-end libraries for each sample. Novaseq 6000 sequence analyser

(Illumina, Inc, San Diego, CA, USA) was employed to carry out multiplex sequencing of the libraries, with four samples per lane at Novogene (Novogene UK Company Limited, Cambridge, United Kingdom), according to the manufacturer's instructions. Pair-end reads of 150 bp were generated.

2.2.2. Bioinformatics analyses

Quality of raw sequencing data was assessed with FastQC (Babraham Bioinformatics, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The quality was measured according to sequence read lengths and base-coverage, nucleotide contributions and base ambiguities, quality scores and over-represented sequences. All the samples passed the quality control (QC) parameters: same length, 100% coverage in all bases, 25% of A, T, G and C nucleotide contributions, 50% GC on base content and less than 0.1% of overrepresented sequences. TrimGalore (Babraham Bioinformatics, http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was used to trim the raw sequences through removing the sequencing adaptor and poly A and T tails, setting default values (stringency of 6 bp) and keeping paired-end reads when both pairs were longer than 40 bp. Hisat2 (Kim *et al.*, 2019) was used to map the filtered reads against the pig reference genome (Sscrofa11.1). After that, HTseq-counts (Anders *et al.*, 2015) was employed to obtain the raw counts for the genes and transcripts and to construct the read counts matrix. Then, the differential expression analyses were carried out using the DESeq2 package (Love *et al.*, 2014) in R environment (Team, 2015). Genes and transcripts were considered as differentially expressed (DEGs) when the $\log_2$ fold change (log2 FC) of the expression differences between the High MB and Low MB groups were lower than -0.58 and higher than 0.58 and with a *p-value* lower than 0.05. The False Discovery Rate was adjusted keeping those DEGs with a *p-adjusted-value* lower than 0.10.

2.2.3. Gene functional classification, network and pathway analyses

The functionality of the DEGs was analysed using Gene Ontology (GO) information. The biological interpretation of the data was performed using FatiGO browser from Babelomics 5 (Babelomics 5, http://babelomics.bioinfo.cipf.es/). STRING tools v11.0 (Szklarczyk *et al.*, 2017) was used to study the potential interactions between

the proteins codified by the DEGs and clustering through the Markov Cluster Algorithm (MCL).

Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Qiagen, CA, USA) bioinformatics tool was used to identify and characterize biological functions, gene networks, canonical pathways and transcription regulatory factors affected by the DEGs. This software assesses the significant association between the data set of DEGs and canonical pathways. In addition, the biological relationships between genes are represented with networks graphs, which it builds with the set of genes using the records harboured in the Ingenuity Pathways Knowledge Base. Potential regulators of differential gene expression were also identified using the tools “upstream regulators” and “causal networks”; these tools analyse whether the potential transcriptional factors and upstream regulators contained in the Ingenuity Knowledge Base repository activate or inhibit the differential gene expression pattern through by estimating a z-score. The z-score statistically measures the significance between the regulator and its potential targets as well as the direction among them (Krämer *et al.*, 2014).

2.3. RNA-seq results validation by quantitative PCR

To perform the technical validation of the RNA-seq experiment, we used RNA samples from the same 12 pigs analysed in the RNA-seq study. We carried out the validation measuring the expression of six genes with quantitative PCR (qPCR); four upregulated in the High or in the Low MB group (*CD209, FES, PLA2G10* and *ZSCAN31*) and two of them not differentially expressed (*DGAT2* and *ATP6*).

Firstly, first-strand cDNA synthesis was carried out using Superscript II (Invitrogen, Life Technologies, Paisley, UK) and random hexamers, in a total volume of 20 µL using 1 µg of total RNA, according to the manufacturer’s instructions. Primer pairs used for quantification were designed using Primer-Blast (NCBI, https://www.ncbi.nlm.nih.gov/tools/primer-blast/) from the available GENBANK and/or Ensembl sequences, covering different exons to assure the amplification of the cDNA. These primer sequences and amplicon lengths is shown in Table S1. Next, a standard PCR on cDNA was performed for each primer to verify amplicon sizes. Then, the quantification was carried out with SYBR Green Mix (Roche, Basel, Switzerland) in a

LightCycler480 (Roche, Basel, Switzerland) and data analysis was performed with LightCycler480 SW1.5 software (Roche, Basel, Switzerland). Three technical replicates were run per each sample and dissociation curves were obtained to confirm the specific amplification of each gene. Four cDNA dilutions were carried out to build a standard curve and estimate PCR efficiency. The statistical analysis was performed using the mean crossing point values (Cp), which is the PCR cycle number when the sample's reaction curve cuts the threshold line. The stability of the endogenous genes *ACTB* and *B2M* was calculated with Genorm (Vandesompele *et al.*, 2002). The relative quantities of DEGs were divided by the geometric means of the two reference genes (as a normalization factor). For the technical validation, we calculated the Pearson correlation between the expression values from RNA-seq and from qPCR, also the concordance correlation coefficient (CCC) between the fold change values from the two techniques was estimated.

3. Results

3.1. Characterization of longissimus dorsi transcriptome and differential expression analyses

The longissimus dorsi transcriptome of the 12 selected pigs was characterised through RNA-seq technique. We obtained a total of 1,498 million raw paired-end reads. After the trimming and filtering processes, 1,497 million reads remained. All samples passed the quality control and 91.80% to 94.07% of the reads were mapped to the porcine reference genome (Sus scrofa 11.1) (Table S2).

A total of 16,746 out of 22,452 genes annotated in the reference genome were detected as expressed in our samples and a total of 17,226 transcripts were expressed. In addition to the genes complying the established filters (|log2 FC| > 0.58 & *p-adjusted-value* <0.10), transcripts fulfilling these filters and belonged to genes not included in the previous dataset were considered as DEGs. The volcano plot (Figure 1a and 1b) represents graphically the expressed genes and transcripts identified. Finally, 57 DEGs were identified in the High and Low MB groups, 53 DEGs were upregulated in the High group (log2 FC ≤ -0.58) while four were upregulated in the Low group (log2 FC ≥ 0.58) (Table S3). Regarding the expression values, log2 FC ranged from -5.42 to 1.26, being the

genes with the highest expression differences between the divergent groups *Carbohydrate Sulfotransferase 1 (CHST1*, log2 FC = -5.42, *p*-value = 2.16 × 10^{-5}, overexpressed in the High group) and *Enoyl-CoA hydratase 1 (ECH1*, log2 FC = 1.26, *p*-value = 1.33 × 10^{-4}, overexpressed in the Low group) (Table S3). Table 1 shows a list of DEGs chosen for their key functions, which would be associated with biological pathways of MB content.

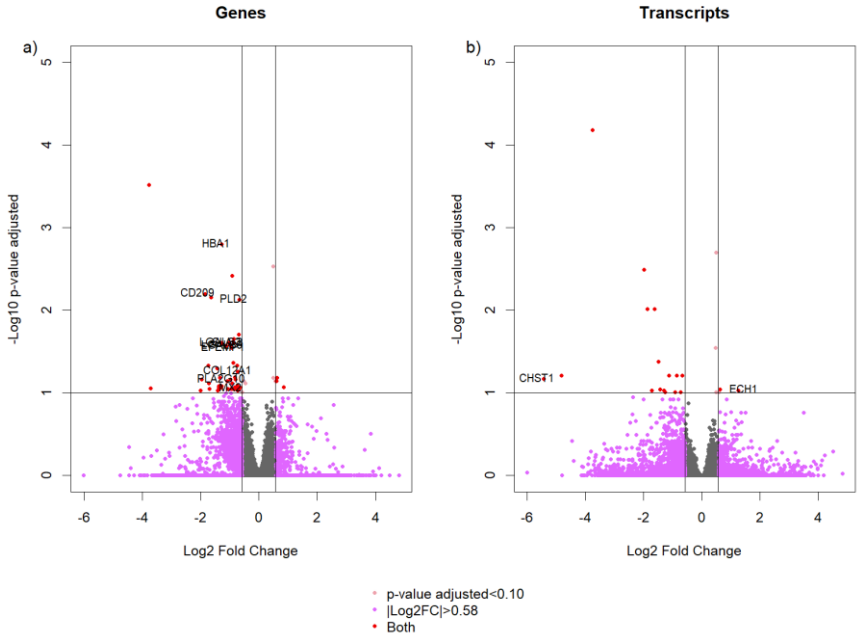


Figure 1. *Volcano plot of differentially expressed genes (DEGs) and transcripts of High MB group vs. Low MB group. Red dots indicate DEGs with q-Value < 0.10 and |Log2 Fold Change| > 0.58. Grey dots are non-significant genes.*

Table 1. Log_2 Fold change, mean expression value in the High and Low myoglobin groups, q-Value and p-Value, corresponding to the most relevant differentially expressed genes (DEGs).

Gene	Log_2 FC	High[a]	Low[a]	*p-Value*	*q-Value*
ECH1	1.257	27.56	66.18	1.33×10^{-04}	0.093
PLD2	-0.676	246.87	153.99	9.13×10^{-06}	0.007
COL12A1	-0.734	286.06	172.33	8.69×10^{-05}	0.055
MX2	-0.824	186.23	104.88	6.74×10^{-04}	0.089
SLA-1	-0.852	14004.76	7759.33	1.00×10^{-05}	0.025
EFEMP1	-0.930	570.68	299.52	4.36×10^{-05}	0.029
PLA2G10	-0.971	91.44	46.57	3.15×10^{-04}	0.069
LGALS9	-0.982	175.18	88.31	3.82×10^{-05}	0.027
LGALS3	-1.031	371.15	181.95	3.22×10^{-05}	0.025
HBA1	-1.285	181.41	73.89	4.97×10^{-07}	0.002
SLA-7	-1.371	328.05	126.35	3.47×10^{-04}	0.085
CD209	-1.857	342.65	94.71	3.44×10^{-06}	0.006
CHST1	-5.418	7.69	0.00	2.16×10^{-05}	0.068

[a] Mean expression values of the High and the Low myoglobin groups are expressed in read counts.

3.2. Gene functional analyses

FatiGO was employed to perform GO enrichment analyses, which recognised 24 GO biological processes (GO_{BP}) and one GO_{SLIM} (cut-down versions of the GO ontologies containing a subset of the terms in GO) enriched in DEGs (Table 2). There was an enrichment of DEGs in processes involved in prostaglandin metabolism. Functional enrichment analyses also showed biological process involved in the metabolism of eicosanoids, oxidation status, fatty acid transport, reactive oxygen species metabolism, T Cell proliferation, cytoskeleton organisation and connective tissue.

The Figure 2 shows the results obtained from STRING, which identified networks of protein-protein interactions codified by annotated DEGs. We found four differentiated clusters comprised proteins codified by DEGs; these proteins are all overexpressed in the High MB group. Cluster 1 is constituted by LGALS9, ENSSSCG00000005055 (LGALS3), SLA-1 and SLA-7 involved in cellular and molecular recognition, moreover LGALS3 is involved in immune metabolism GO_{BP} (Table 2). Cluster 2 is constituted by TRIM6, MX2, IFI44 and IFI44L involved in defence against virus. Cluster 3 is constituted by EPB41L1 and EPB41L3, associated with actin binding and actomyosin and cytoskeleton structure organization. Lastly, cluster 4 is constituted by EFEMP1, MFAP2 and FBLN1, associated with cell function, cell adhesion and degradation of the extracellular matrix.

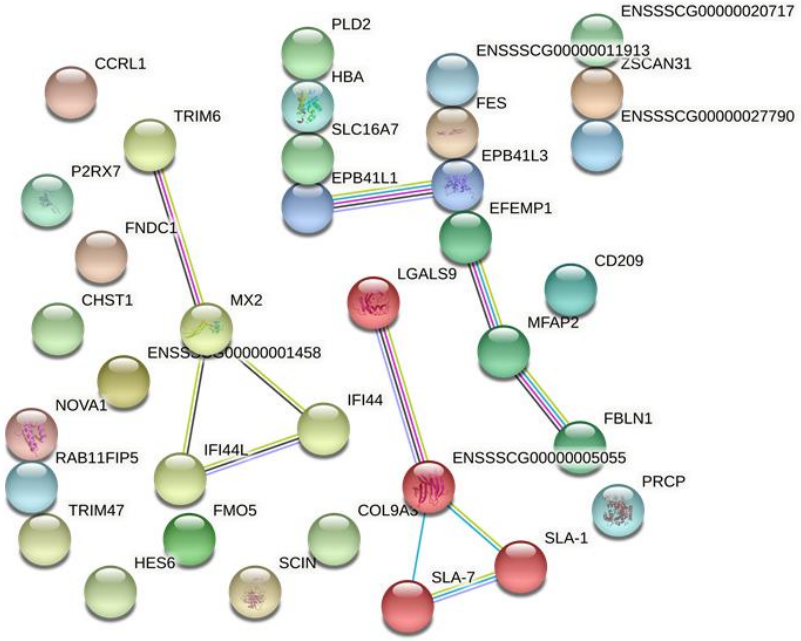


Figure 2. *Network of protein-protein interactions predicted with STRING database. Same colour nodes sharing multiple edges are grouped in the same cluster.*

Table 2. List of significantly overrepresented Gene Ontology (GO) terms related to myoglobin content on differentially expressed genes (DEGs) using FatiGO.

Term	Genes	Adjusted *p*-Value
GO_{BP}		
Prostaglandin secretion (GO:0032310)	*P2RX7, PLA2G10*	2.55×10^{-02}
Positive regulation of icosanoid secretion (GO:0032305)	*P2RX7, PLA2G10*	2.55×10^{-02}
Regulation of prostaglandin secretion (GO:0032306)	*P2RX7, PLA2G10*	2.55×10^{-02}
Positive regulation of prostaglandin secretion (GO:0032308)	*P2RX7, PLA2G10*	2.55×10^{-02}
Mitochondrial depolarization (GO:0051882)	*P2RX7, IFI6*	2.55×10^{-02}
Regulation of mitochondrial depolarization (GO:0051900)	*P2RX7, IFI6*	2.55×10^{-02}
Prostaglandin transport (GO:0015732)	*P2RX7, PLA2G10*	2.55×10^{-02}
Positive regulation of fatty acid transport (GO:2000193)	*P2RX7, PLA2G10*	2.58×10^{-02}
Regulation of icosanoid secretion (GO:0032303)	*P2RX7, PLA2G10*	3.37×10^{-02}
Monocarboxylic acid transport (GO:0015718)	*P2RX7, PLA2G10, SLC16A7*	3.37×10^{-02}
Positive regulation of organic acid transport (GO:0032892)	*P2RX7, PLA2G10*	3.37×10^{-02}
Regulation of fatty acid transport (GO:2000191)	*P2RX7, PLA2G10*	3.97×10^{-02}
Reactive oxygen species metabolic process (GO:0072593)	*P2RX7, PRCP, HBA1*	3.97×10^{-02}
T cell proliferation (GO:0042098)	*P2RX7, CD209, LGALS3*	3.97×10^{-02}
Cartilage development (GO:0051216)	*EFEMP1, SCIN, MGP*	3.97×10^{-02}
Cortical actin cytoskeleton organization (GO:0030866)	*EPB41L3, EPB41L1*	3.97×10^{-02}
Positive regulation of ion transport (GO:0043270)	*P2RX7, PLA2G10, LGALS3*	3.97×10^{-02}
Positive regulation of cytoskeleton organization (GO:0051495)	*P2RX7, SCIN, FES*	3.97×10^{-02}
Cortical cytoskeleton organization (GO:0030865)	*EPB41L3, EPB41L1*	4.25×10^{-02}
Icosanoid secretion (GO:0032309)	*P2RX7, PLA2G10*	4.65×10^{-02}
Icosanoid transport (GO:0071715)	*P2RX7, PLA2G10*	4.65×10^{-02}
Fatty acid derivative transport (GO:1901571)	*P2RX7, PLA2G10*	4.65×10^{-02}
Connective tissue development (GO:0061448)	*EFEMP1, SCIN, MGP*	4.65×10^{-02}
Phosphatidylglycerol metabolic process (GO:0046471)	*PLA2G10, PLD2*	4.65×10^{-02}
GO_{slim}		
Proteinaceous extracellular matrix (GO:0005578)	*EFEMP1, MGP, FBLN1*	1.04×10^{-02}

The additional functional analysis performed with IPA software revealed four networks enriched in DEGs (Table 3). IPA assigns a network score in concordance to the size of the network and the number of target genes involved. This score is estimated as the negative logarithm of the *p*-value calculated by Fisher's exact test. The most relevant function represented in network 1 was *Immunological disease* (Figure 3), in network 2 *Lipid Metabolism* (Figure 4), in network 3 *Hematological System* (Figure S1) and in network 4 *Lipid Metabolism* (Figure S2).

Table 3. Relevant enriched networks and functions related to myoglobin content identified in the set of DEGs between the High and Low groups identified by IPA software. Genes showing the highest expression differences between groups are in bold.

ID	Molecules in Network	Score	Focus Molecules	Functions
1	**ACKR2, CD209**, chemokine, **COL12A1**, Collagen(s), **EFEMP1**, elastase, ERK1/2, **FBLN1, FES**, **HBA1/HBA2**, hemoglobin, HES6, **IFI44, IFI44L, IFI6**, IFN Beta, Ige, IgG, Immunoglobulin, Interferon alpha, JAK, **LGALS3, LGALS9, MGP, MX2, P2RX7, PLA2G10, PLD2, PRCP**, Pro-inflammatory Cytokine, **RAB11FIP5, SLC16A7**, TCR, Tgf beta	54	21	Dermatological Diseases and Conditions, Immunological Disease, Organismal Injury and Abnormalities
2	**BASP1, beta-estradiol, BICD1, CCDC191, CEACAM5, CHST1**, chymotrypsin, CYLD, D-glucose, DDIT3, **DEPDC1B, ECH1**, EGFR, ESR1, ESR2, **FAM160A1, FMO5**, FNDC1, G protein beta gamma, GIPC1, **GTPBP6**, HRAS, IL1B, **MFAP2**, NAB1, NOTCH1, ONECUT2, PLEK2, PRSS35, PTPN11, **SH3BGRL3**, SRC, sulfotransferase, TBK1, TRIM47, **VSTM1**, **ZSCAN31**	35	15	Cell Cycle, Gene Expression, Lipid Metabolism
3	**ACKR4**, Actin, ADGRG3, Akt, Anti-inflammatory Cytokine, C1GALT1C1, caspase, CCL27, Cyb5r3, cysteinyl-leukotriene, **EPB41L1, EPB41L3**, ERK, estrogen receptor, Histone h3, HTR1B, ICAM2, ICAM3, JAM2, lewis Y, mannan, mannose, **MAPK1**, NFkB (complex), NLRC4, NOVA1, NRG3, P38 MAPK, Pak, **RPRM, SCIN**, SPINK7, STMN4, **TRIM34**, Vegf	14	7	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking
4	ANGPTL3, ANGPTL4, ANGPTL8, APOA5, **GPIHBP1**, Immunoglobulin, LPL, oleic acid	2	1	Cardiovascular Disease, Lipid Metabolism, Small Molecule Biochemistry

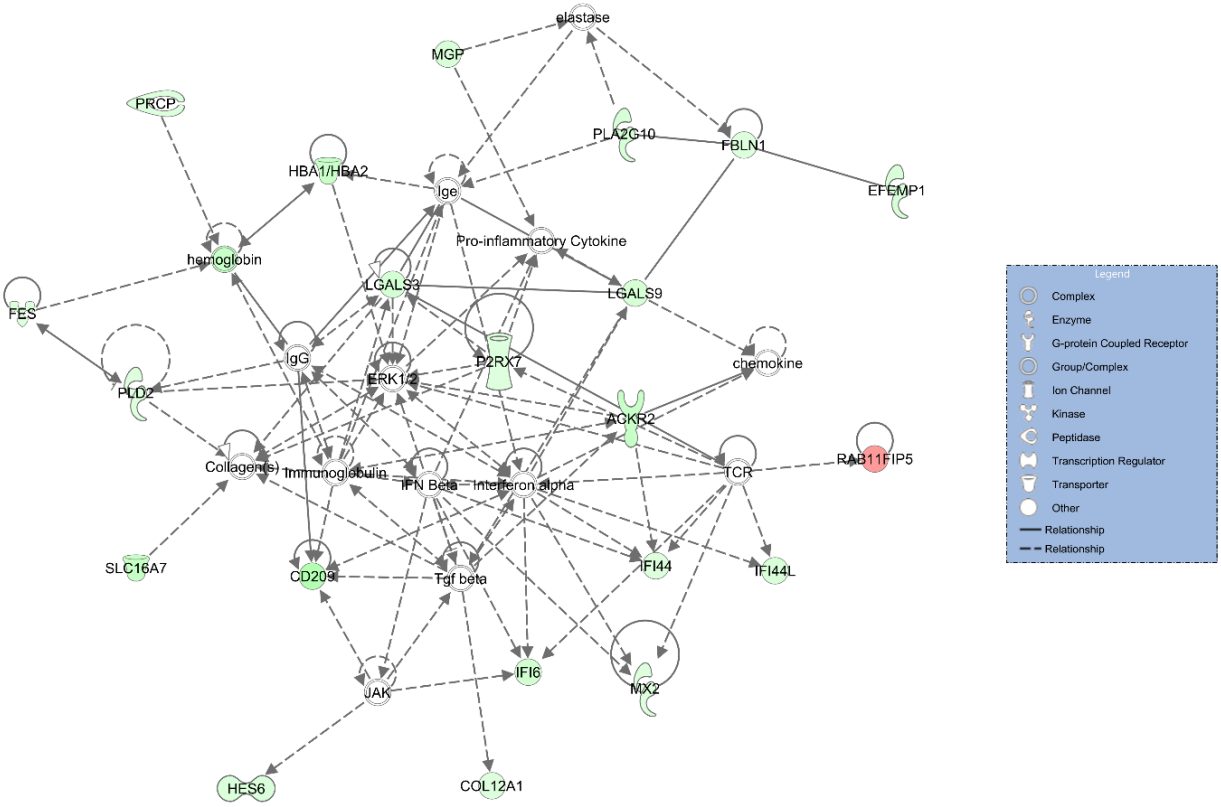


Figure 3. Gene network #1: Dermatological Diseases and Conditions, Immunological Disease, Organismal Injury and Abnormalities. Genes up-regulated and down-regulated in the High MB group are represented in green and red colours, respectively.

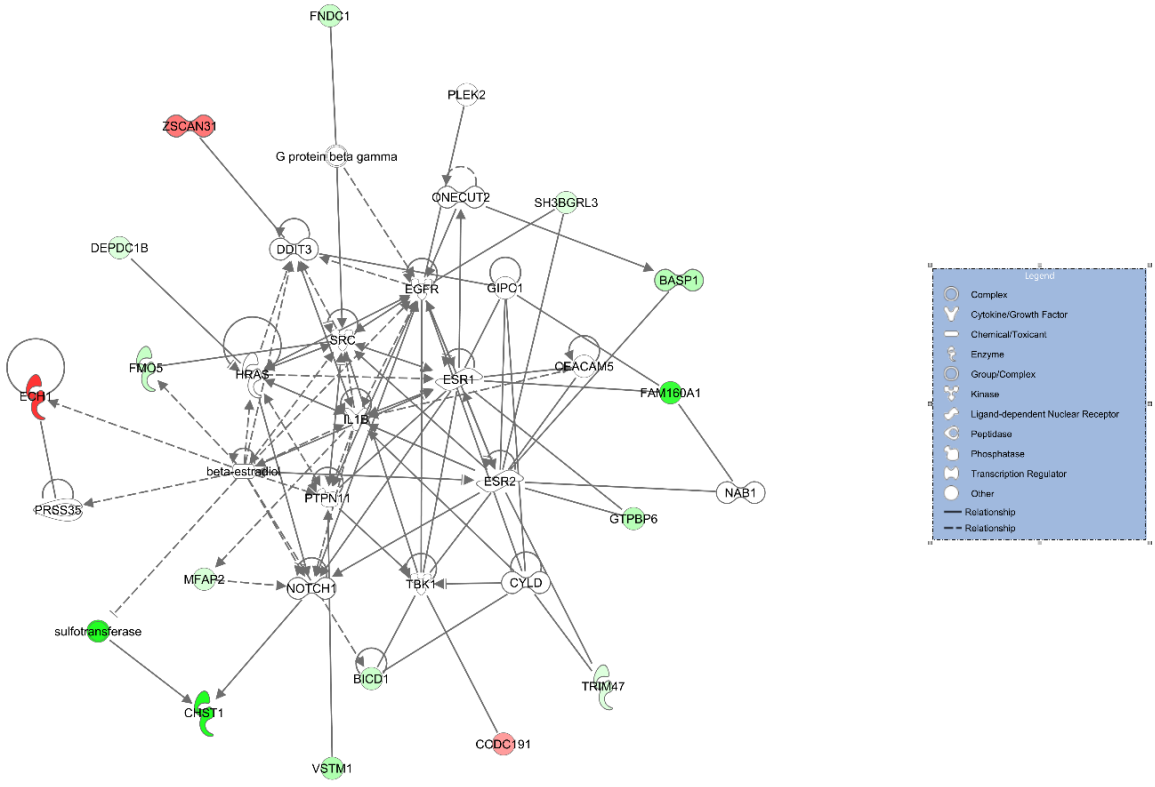


Figure 4. Gene network #2: Cell Cycle, Gene Expression, Lipid Metabolism. Genes up-regulated and down-regulated in the High MB group are represented in green and red colours, respectively.

3.2.1. Canonical pathways analysis

We carried out a functional interpretation of global gene expression differences using IPA canonical pathway analysis. Regarding the dataset of 57 DEGs, a total of four canonical pathways were significantly enriched (*p*-value < 0.05, Table 4). The most significant pathway was *Phospholipases* (*p*-value = 0.007) and the other three relevant pathways were *Antioxidant action of vitamin C*, *Choline biosynthesis III* and *Inflammasome pathway*. All the molecules included in these pathways are overexpressed in the High MB group. Nevertheless, the functional analysis did not report any pathway with assigned z-score, therefore none of them were predicted for an overall activation or inhibition in the High or Low MB groups.

Table 4. List of significant pathways (*p*-Value < 0.05) identified in the set of DEGs according to the High and Low myoglobin groups identified by Ingenuity Pathway Analysis (IPA) software[1].

Canonical Pathways	*p*-Value	Ratio	Molecules
Phospholipases	0.007	0.031	PLA2G10, PLD2
Antioxidant Action of Vitamin C	0.019	0.018	PLA2G10, PLD2
Choline Biosynthesis III	0.028	0.067	PLD2
Inflammasome pathway	0.038	0.050	P2RX7

Ratio: number of DEGs in a pathway divided by the number of genes comprised in the same pathway. [1]IPA not predicted any assigned z-Score value for the significant pathways.

3.2.2. Transcription regulatory factors

The IPA upstream analysis and regulator effect tools were used to determine the potential transcription regulatory factors of DEGs involved in molecular processes, which may explain the differential expression noticed among the High and Low MB groups. In this study, a total of 156 transcriptional regulators were found (*p*-value < 0.05, Table S4). The direction of the activation state of five regulators was statistically predicted (z-score > 2 or z-score < -2, Table 5). PRL, IFNG and IRF7 were predicted as activated in the High MB group (z-score < -2) while IL1RN and MAPK1 were activated in the Low MB group (z-score > 2). In addition to this, SPI1 (*p*-value = 2.63×10^{-4}, Table S4) presented a negative z-score (-1.98), indicating a trend for activation in the High MB group.

Table 5. List of significant upstream regulators identified in the set of DEGs according to the High and Low myoglobin groups (*p*-Value < 0.05 and z-Score > 2 or < -2).

Upstream Regulator	Molecule Type	PAS	Activation z-Score	*p*-Value of overlap	Molecules in Dataset
PRL	cytokine	High Mb group	-2.219	2.35×10^{-04}	IFI44, IFI44L, IFI6, MGP, MX2
IFNG	cytokine	High Mb group	-2.038	7.49×10^{-03}	CD209, FBLN1, IFI44, IFI44L, IFI6, LGALS3, LGALS9, MX2
IRF7	transcription regulator	High Mb group	-2	3.61×10^{-04}	IFI44, IFI44L, IFI6, MX2
IL1RN	cytokine	Low Mb group	2.236	5.78×10^{-06}	IFI44, IFI44L, IFI6, LGALS9, MX2
MAPK1	kinase	Low Mb group	2.646	9.72×10^{-06}	FES, HBA1/HBA2, IFI44, IFI6, LGALS3, MX2, TRIM34

Furthermore, a set of master regulators were statistically predicted (Table 6), where four were activated in the High MB group (z-score < -2; SPI1, PRL, IRF7, IFNA2) and another four in the Low MB group (MAPK1, IL1RN, MECP2, Hnf3). A complementary functional analysis with IPA was carried out considering those genes with a *p-adjusted-value* lower than 0.20. With this data set, one regulatory effect network was predicted (Figure 5), representing a causal hypothesis to interpret the potential mechanism of the master regulator (SPI1) in the expression of some DEGs.

3.3. RNA-seq validation by qPCR

In order to validate the RNA-seq results, we calculated the Pearson correlation with the quantification of the expression of six genes using qPCR in the same 12 samples. Likewise, the corresponding *p*-values and CCC were obtained (Table 7). Three genes showed a correlation coefficient higher than 0.75. Five genes presented a significant *p*-value (<0.05). The CCC was equal to 0.951, suggesting a substantial concordance between RNA-seq and qPCR expression values (Miron *et al.*, 2006). *CD209* and *FES* genes showed the highest and the lowest concordance between methods, respectively.

Table 6. Ingenuity Pathway Analysis (IPA). List of significant Master regulators (p-value < 0.05) with assigned z-Score identified in the set of DEGs according to High and Low myoglobin groups.

Master Regulator	Molecule Type	Participating regulators	PAS	Activation z-score	p-Value of overlap	Target Molecules in Dataset
MAPK1	kinase	MAPK1	Low Mb group	2.646	2.52×10^{-06}	FES, HBA1/HBA2, IFI44, IFI6, LGALS3, MX2, TRIM34
IL1RN	cytokine	IL1RN	Low Mb group	2.236	5.16×10^{-06}	IFI44, IFI44L, IFI6, LGALS9, MX2
PRL	cytokine	PRL	High Mb group	-2.236	1.99×10^{-04}	IFI44, IFI44L, IFI6, MGP, MX2
IRF7	transcription regulator	IRF7	High Mb group	-2	3.15×10^{-04}	IFI44, IFI44L, IFI6, MX2
MECP2	transcription regulator	MECP2, SPI1	Low Mb group	2.236	3.56×10^{-04}	CD209, HBA1/HBA2, IFI44, IFI44L, IFI6
IFNA2	cytokine	IFNA2	High Mb group	-2	5.91×10^{-04}	IFI44, IFI44L, IFI6, MX2
SPI1	transcription regulator	SPI1	High Mb group	-2	6.14×10^{-04}	CD209, IFI44, IFI44L, IFI6
Hnf3	group	estrogen receptor, FOXA1, FOXA2, FOXA3, Hnf3, IRF3, STAT5B	Low Mb group	2.646	1.68×10^{-03}	COL12A1, FBLN1, IFI44, IFI44L, IFI6, MGP, RPRM

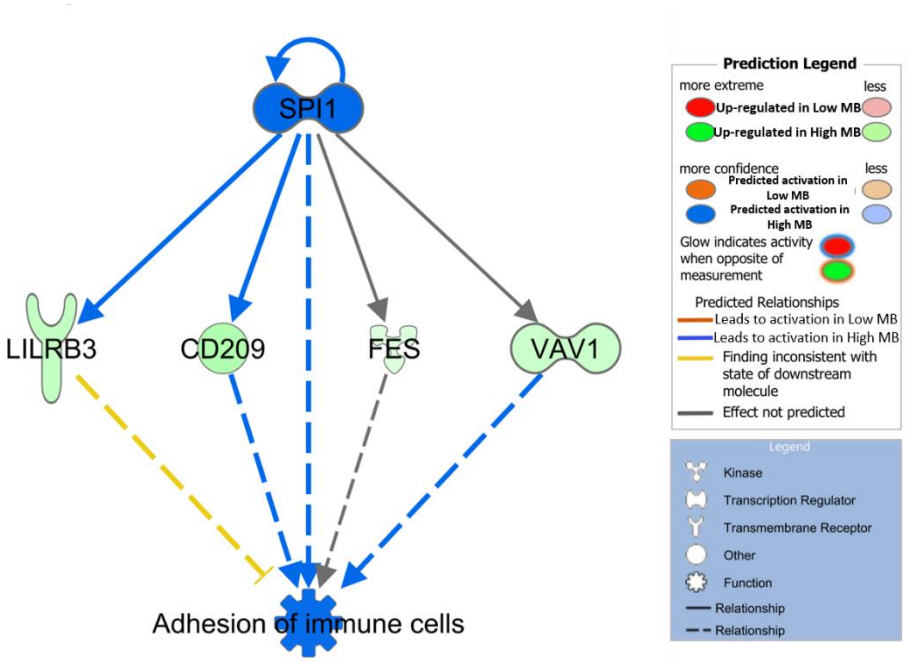


Figure 5. Master regulator effects network predicted in Iberian pigs fattened in an open-air free-range system.
In the upper tier is SPI1 (predicted to be activated, blue colour). In the middle, there are the genes whose expression changes in response to the activation of SPI1 (green upregulated for the High MB group). Dashed lines between SPI1 and DEGs represent the interactions, predicted to be activated (blue lines) or predicted to be inhibited (orange lines). In the lower tier, the expected phenotypic activate function (adhesion of immune cells, blue colour) is shown.

Table 7. Technical validation of RNA-seq results by quantitative PCR (qPCR). Fold Change values (FC), Pearson correlations (r^2) and Concordance Correlation Coefficient (CCC) between expression values obtained from both techniques.

Gene	Expression type	qPCR Log_2 FC	RNAseq Log_2 FC	r^2	*p*-value	CCC
CD209	H>L	-1.200	-1.857	0.99	6.39×10^{-10}	0.951
FES	H>L	-0.211	-0.817	0.42	0.17	
PLA2G10	H>L	-0.964	-0.971	0.62	0.041	
ZSCAN31	L>H	0.835	0.844	0.59	0.043	
DGAT2	NO DE	-0.540	-0.852	0.90	5.92×10^{-5}	
ATP6	NO DE	-0.009	-0.087	0.75	5.18×10^{-3}	

4. Discussion

Myoglobin (MB) content is related to the red colour of meat and it is directly associated with consumer preferences regarding meat consumption. In the current study, transcriptome analysis between divergent Iberian pigs for breeding values of myoglobin content showed 57 DEGs and a set of functional pathways and protein

networks in which they are involved. These results provide more insight on the mechanisms of the processes underlying this trait.

The first expected result is that the gene encoding myoglobin was differentially expressed. However, this gene was not annotated in the version of the pig reference genome used in the present study (Sscrofa11.1). An improved annotation of the reference genome would reveal whether this gene is differentially expressed in the present experiment. However, *Hemoglobin subunit alpha 1 (HBA1)* encoding alpha-globin, which is a component of hemoglobin in charge of carrying oxygen to cells and tissues all over the body, is one of the overexpressed DEG in the High MB group. The functional analysis revealed that *HBA1* play a role in the metabolic process of reactive oxygen species (ROS) (GO:0072593, Table 2). The function and regulation of non-erythrocyte hemoglobin is not fully understood, however oxidative stress seems to be associated with higher hemoglobin expression in cells other than erytrhocyte (Liu *et al.*, 2011). In addition, the myoglobin and hemoglobin content in striated muscles is correlated in several species, including pigs (O'Brien *et al.*, 1992), and these authors associated the hemoglobin content with a greater aerobic capacity of the tissue and its blood flow, and the myoglobin content with high physical activity. Therefore, a higher expression of hemoglobin could protect against oxidative stress.

Several of the DEGs found in the current study such as *phospholipase A2 group X* (*PLA2G10), phospholipase D2 (PLD2)* (both overexpressed in the High MB group) and *ECH1* (overexpressed in the Low MB group) are involved in lipid metabolism. *PLA2G10* gene encodes for a lipolytic enzyme, which plays a role in lipid pathways such as the hydrolysis of cell membrane phospholipids and the release of free fatty acids and lysophospholipids (Vadas *et al.*, 1986, Murakami *et al.*, 2020). According to the functional enrichment analysis, *PLA2G10* gene is involved in regulation of prostaglandin secretion and transport (GO:0032306, GO:0032308, GO:0015732), fatty acid transport (GO:2000193, GO:2000191) and regulation of eicosanoid secretion (GO:0032303, GO:0032305) (Table 2). The enzyme has a role maintaining membrane phospholipid homeostasis (Sun *et al.*, 2010), as well as its function is important in inflammation since it releases arachidonic acid, a precursor of eicosanoids (Hanasaki *et al.*, 2002). In a recent review, Murakami *et al.* (2020) suggested that *PLA2G10* gene is involved in

immunological functions, such as anti-inflammatory phenotypes. Furthermore, this enzyme acts as an important mechanism of defence against intestine parasites and viruses, indicating that it has a role in adaptive and innate immune responses. While PLA2G10 has been used as an inflammatory marker, MB has been used as tissue injury marker (Tartibian *et al.*, 2011) because high levels are related to a high vulnerability of the membrane (Driessen-Kletter *et al.*, 1990). Therefore, this gene would play a double key function all at once: lipid and immune metabolism. In agreement to this, several authors have already described a connection between both functions (Hubler et al., 2016; Gianfrancesco et al., 2019; Batista-Gonzalez et al., 2020). In fact, these studies described that lipid metabolism plays a role in regulating immune cells activation.

The *PLD2* gene codifies an enzyme that plays a pivotal role in the regulation of cell function and cell fate (Liscovitch *et al.*, 2000). The functional analysis revealed that this gene is involved in *Choline biosynthesis III* pathway (Table 4). In the same way, Yang *et al.* (2004) described that PLD2 hydrolyses phosphatidylcholine from the cell membrane generating phosphatidic acid, which is a lipid messenger that mediates signalling functions. Phosphatidic acid has been reported to be involved in the iron-induced synaptic response (Mateos *et al.*, 2012) and it is well known that myoglobin is one of the main iron deposits in mammals, therefore a higher myoglobin content could be related to higher iron releases and, consequently, higher activation of *PLD2*.

The third DEG involved in lipid metabolism, *ECH1,* is one of the few down-regulated in the High MB group and the only one gene presented in Table 1. This gene encodes an enzyme that hydrates short- and medium-chain enoyl-CoA and is related to upregulation of β-oxidation (Bahnson *et al.*, 2002). Lower expression of this gene was also observed in indigenous Chinese pigs compared to Yorkshire, which also showed better meat quality parameters, such as color, than Yorkshire pigs (Li *et al.*, 2016). Our results should point that muscles with higher myoglobin content could have lower β-oxidation, which is a different process from the membrane lipid oxidation mentioned above.

The oxidation-reduction mechanisms are closely related to the generation of the four redox states of myoglobin (DMB, OMB, MetMB and COMB), which take place mainly within the mitochondria. Antioxidants and secondary reactive products of lipid

oxidation (ROS) are well known to influence the colour stability of pork, affecting the a* parameter and rancidity (Faustman *et al.*, 2010, Suman *et al.*, 2013, Li *et al.*, 2016). Moreover, an excessive amount of ROS possibly damages the mitochondria and cells due to interactions with proteins, lipids and acids nucleic, also an increase in the oxidation of mitochondrial fatty acid could increase DNA damage (Cooke *et al.*, 2003). The processes involved on lipid oxidation such as fatty acid elongation and biosynthesis of unsaturated fatty acids also affect meat colour and rancidity (Wood *et al*., 2008; Li *et al.*, 2016). The reactive products of lipid oxidation compromise meat colour by precipitating MB oxidation contributing to meat discoloration, while antioxidants such as Vitamin C and E play a protection role and enhance meat colour (redness) and stability of lipids (Faustman *et al.*, 2010, Suman *et al.*, 2013). In the same way, *Antioxidant action of vitamin C* (Table 4) is one of the most significant pathways in our study, where two of the overexpressed DEGs are involved on LD muscle of pigs with higher MB content (*PLA2G10* and *PLD2*). Li *et al.* (2016) found that DEGs implicated in the metabolism of lipid oxidation could promote myoglobin oxidation, thereby accentuating cited dark brown colour of the meat. Therefore, strategies focused on restrain lipid oxidation can reduce rancidity and improve colour stability.

Several genes involved in the innate *(MX2*) and adaptive immune response (*CD209, SLA1, SLA7, LGALS3 and LGALS9*) and the inflammatory response (*CHST1*) were also differentially expressed between the High and Low MB groups. While the innate immune system acts as the first line of defence, the adaptive immune system represents the second line, in which T-lymphocytes (T-cells) and others cells are involved. The adaptive system provides protection and produces antigen-specific antibodies from pathogens (Hubler *et al.*, 2016). *MX Dynamin Like GTPase 2 (MX2*) encodes a protein that has been recognised in the immune defence resistance to virus infection and in the response to interferon alpha (King *et al.*, 2004, Goujon *et al.*, 2013, Kane *et al.*, 2013). Some studies have described the pivotal antiviral activity also in pigs (Sasaki *et al.*, 2014, Albarracín *et al.*, 2017). A differential expression of the *MX2* gene was observed in a study that compared the transcriptome between goat breeds with different meat characteristics, being higher in the breed with better meat quality attributes (Shen *et al.*, 2021).

DC-SIGN (CD209) encodes a protein with a role in the regulation of T-cells proliferation (Ryan *et al.*, 2002). In the same way, our functional analyses revealed that *CD209* is associated with T-cell proliferation biological process (GO: 0042098, Table 2). A recent genomic study in Iberian pigs proposed *CD209* as a good candidate gene for its association with immune defence and modulation during infection by pathogens (Alonso *et al.*, 2020) and it was overexpressed in Duroc pigs with high contents of intramuscular fat and saturated and monounsaturated fatty acids in the gluteus medius muscle (Cardoso *et al.*, 2018). Increased expression of the *CD209* gene has been observed in skin biopsies from German shepherd dogs with atopic dermatitis and a relationship between this CD209 protein and inflammation has been suggested (Tengvall et al 2020).

Src like adaptor 1 and 7 (*SLA-1* and *SLA-7*) genes belong to a family that encode proteins of the porcine Major Histocompatibility Complex. *Galectin 3* and *Galectin 9* (*LGALS3* and *LGALS9*) are S-type lectins with affinity for beta-galactoside that have antimicrobial activity against bacteria and fungi. In our functional transcriptome study *LGALS3* was involved in T-cell proliferation GO_{BP} (Table 2) and is also implicated in the IPA network 1 related to *immunological disease* function (Table 3). Moreover, a porcine transcriptome study recognised *LGALS3* as a relevant innate immune gene expressed in healthy pigs (Snyman *et al.*, 2014). Besides the function in antigen recognition, the CCGAAG haplotype detected in *LGALS9* has been proposed as a potential marker for intramuscular fat selection in loin muscle (Li *et al.*, 2010). Finally, *Carbohydrate Sulfotransferase 1* (*CHST1*), which is overexpressed in High MB group and shows the greatest differences in expression levels (Table 1). This gene encodes a member of the keratin sulfotransferase family of proteins that catalyses the sulfation of the proteoglycan keratin and generates L-selectin ligands which are pro-inflammatory agents (Li et al, 2001).

Other two DEGs overexpressed in the High MB group are *EFEMP1* and *COL12A1*. *EFEMP1* encodes a glycoprotein of the extracellular matrix, EGF-containing fibulin-like extracellular matrix protein 1. *EFEMP1* is involved in GO_{BP} related to cartilage and connective tissue development. This glycoprotein is part of the fibulin family, which are proteins that modulate cell morphology, growth, adhesion and motility (Gallagher et al 2005). Transcriptome and methylome analyses in different pig breeds have suggested

that *EFEMP1* is involved in growth and developmental processes (Puig-Oliveras *et al.*, 2014, de Yang *et al.*, 2016, Hou *et al.*, 2020) and has also been associated with higher human height (Kemper *et al.*, 2012). This gene has also been described as a regulator of hypoxia in Tibetan pigs (Jia *et al.*, 2016). On the other hand, SNPs effects of *EFEMP1* in oleic acid have been reported in Wagyu x Angus beef (Zhang *et al.*, 2012); however, no effect of this gene in oleic acid has been observed in pigs. Collagen proteins are the elementary constituent of extracellular matrix. *Collagen Type XII Alpha 1 Chain (COL12A1)* belongs to the collagen family of genes and encodes a protein that plays a key role in organizing the structure of the extracellular matrix and fibrils of collagen. An upregulation of the *COL12A1* gene has been observed when the expression in muscle was compared between crossbred Duroc x Iberian with Iberian piglets (Óvilo *et al.*, 2014). Furthermore, Duroc x Iberian crossbreed pigs have less myoglobin content than purebred Iberian pigs (Clemente *et al.*, 2012). Our results indicated an upregulation of the *COL12A1* gene in the High MB group, which is somehow contradictory respect the cited studies, however, it is worth to mention that the current study was carried out in Iberian pigs with an average slaughter age of 17 months and the Óvilo *et al*. (2014) authors analysed the transcriptome of piglets with 28 days.

The upstream analysis predicted a series of regulatory factors that are not necessary differentially expressed in the studied muscle tissue. Several of these regulators (MAPK1, IL1RN, PRL and SPI1) were identified as master regulators (Table 6). A master regulator is a molecule at the top of a regulatory hierarchy and is expressed at the inception of a developmental lineage, participating in the regulation of multiple downstream genes (Chan and Kyba, 2013). One of the most important factors recognized as a master regulator is mitogen-activated protein kinase 1 (MAPK1), which has been recognized as a transcriptional regulator involved in the differentiation of porcine myocytes and interrupting the development of adipocytes (Wang *et al.*, 2017), as well as in cellular pathways such as proliferation, differentiation, transcription, cell motility and apoptosis (Nishida *et al.*, 1993, Vomastek *et al.*, 2008). This regulator was predicted to be activated in meat samples with lower MB content, regulating the expression of the *FES, HBA1, LGALS3* and *MX2* genes, among other.

Spi-1 Proto-Oncogene (SPI1) master regulator was activated in the High MB group (Table 6). Figure 5 represents a causal network hypothesis that could explain the

regulatory mechanism of SPI1 on four genes *CD209, FES, LILRB3* and *VAV1*. *SPI1* would activate the expression of *CD209* that is overexpressed in the High MB group, which it has been related previously with immune defence and inflammation. This protein regulates the expression of multiple genes involved in the immune metabolism (Gangenahalli *et al.*, 2005); also, *SPI1* gene has been related to the regulation of B-cells (B lymphocytes), T-cell and myeloid (Imoto *et al.*, 2010). Therefore, an overexpression of *SPI1* would promote adhesion of the immune cells' mechanism. Moreover, the *SPI1* gene has been related to the regulation of adipogenesis in porcine transcriptome studies (Li *et al.*, 2011, Wei *et al.*, 2015), thus, the relationship between lipid metabolism and immune system was again observed.

The results shown here point out that the Iberian pigs with high myoglobin content have an overexpression of genes codifying pro-inflammatory proteins. Genes involved in lipid metabolism pathways were also related to immune system, which reinforce the possibility for a double key function of these DEGs. According to our results, animals with higher myoglobin content seem to have activated lipid oxidation, and, we could hypothesize that an increase in oxidation would induce the hemoglobin gene expression and augment the myoglobin content in longissimus dorsi muscle, since, as it has been suggested, hemoglobin and myoglobin have a protective effect against oxidation. However, further functional studies should be carried out to support this evidence.

5. Conclusions

In this study, a total of 57 differentially expressed genes were identified on the transcriptome of LD muscle in Iberian pigs with divergent breeding values for myoglobin content. Functional analyses carried out have revealed that DEGs codify proteins involved in processes related with inflammation, lipogenesis and immune defence. These results suggest that pro-inflammatory proteins, involved in lipid oxidation, could be inducing the expression of *HBA1* gene and increasing the myoglobin content since both, hemoglobin and myoglobin content have a protective effect against oxidative stress. Polymorphisms located in regulatory regions of the cited DEGs could be associated with their altered expression and, therefore, they are could be used in marker assisted selection. Finally, the most promising candidate gene underlying myoglobin content variation seems to be *HBA1* and further studies searching for polymorphism mapped in this gene and association studies with myoglobin content should be carried out.

Acknowledgements

We want to thank to Sánchez Romero Carvajal (SRC) enterprise for the technical support, especially to its manager Jose María Pariente, Fernando Gómez-Carballar and Fernando Sánchez-Esquiliche.

Conflict of interest

The authors Luisa Ramírez and Gema Matos are employees of the enterprise "Sánchez Romero Carvajal" and Miguel Ángel Fernández-Barroso, Yolanda Núñez, Juan María García-Casco and María Muñoz were employees of the "Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria—INIA" when the study was carried out. Therefore, we declare no conflicts of interest regarding the writing of this manuscript.

Funding

This work was financially supported by CON15-078, CON17-125 (INIA-SRC) and IDI-20171141 (CDTI) grants. Miguel Ángel Fernández-Barroso was funded by a FPI Ph.D. grant from the INIA institution.

References

Albarracin L, Kobayashi H, Iida H, Sato N, Nochi T, Aso H, Salva S, Alvarez S, Kitazawa H & Villena J. (2017) Transcriptomic Analysis of the Innate Antiviral Immune Response in Porcine Intestinal Epithelial Cells: Influence of Immunobiotic Lactobacilli. *Frontiers in Immunology*. 8:57. doi: 10.3389/fimmu.2017.00057.

Alberti P., Panea B., Ripoll G., Sañudo C., Olleta J. L., Hegueruela I, & Serra X. (2005) Medición del color. In Estandarización de las metodologías para evaluar la calidad del producto (animal vivo, canal, carne y grasa) en los rumiantes. Serie Ganadería Nº 3. (pp. 216-225). Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid (Spain).

Alonso I., Ibáñez-Escriche N., Noguera J., Casellas J., Martín de Hijas-Villalba, M. Gracia-Santana M. & Varona L. (2020) Genomic differentiation among varieties of Iberian pig. *Spanish Journal of Agricultural Research* **18** (1), e0401. doi: http://dx.doi.org/10.5424/sjar/2020181-15411.

Alves E., Óvilo C., Rodríguez M.C. & Silió L. (2003) Mitochondrial DNA Sequence Variation and Phylogenetic Relationships among Iberian Pigs and Other Domestic and Wild Pig Populations. *Animal Genetics* **34**, 319–324.

Anders S., Pyl P.T. & Huber W. (2015) HTSeq—A Python framework to work with high-throughput sequencing data. *Bioinformatics*. **31**, 166–169.

Bahnson B.J. Anderson V.E. & Petsko G. A. (2002) Structural mechanism of enoyl-CoA hydratase: three atoms from a single water are added in either an E1cb stepwise or concerted fashion. *Biochemistry* **41**, 2621–2629.

Batista-Gonzalez A., Vidal R., Criollo A., & Carreño L.J. (2020) New Insights on the Role of Lipid Metabolism in the Metabolic Reprogramming of Macrophages. *Frontiers in Immunology*. 10:2993. doi: 10.3389/fimmu.2019.02993.

Cardoso T.F., Quintanilla R., Castelló A., González-Prendes R., Amills M. & Cánovas Á. (2018) Differential expression of mRNA isoforms in the skeletal muscle of pigs with distinct growth and fatness profiles. *BMC Genomics* **19**, 145. https://doi.org/10.1186/s12864-018-4515-2.

Chan S.S.K. & Kyba M. (2013) What is a Master Regulator? *Journal Stem Cell Research & Therapy*. 3: e114. doi:10.4172/2157-7633.1000e114.

Clemente I., Polvillo O., Juárez M., Avilés C., Membrillo A., Horcada A., Diéguez E., Racero J.C., Molina A. (2012) Comparison of pork quality from pure and crossbred Iberian pig. in De Pedro E.J. (ed.), Cabezas A.B. (ed.). In proceedings 7th International Symposium on the Mediterranean Pig, Zaragoza. CIHEAM Options Méditerranéennes: Série A. Séminaires Méditerranéens. 101. (441-446).

Cooke M.S., Evans M.D., Dizdaroglu M. & Lunec J. (2003) Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB Journal* **17**:1195–1214.

Driessen-Kletter M.F., Amelink G.J., Bär P.R., van Gijn J. (1990) Myoglobin is a sensitive marker of increased muscle membrane vulnerability. Journal Neurology **237**, 234–238

Fabuel E., Barragán C., Silió L., Rodríguez M.C. & Toro M.A. (2004) Analysis of Genetic Diversity and Conservation Priorities in Iberian Pigs Based on Microsatellite Markers. *Heredity* **93**, 104–113.

FastQC. Available online: "http://www.bioinformatics.babraham.ac.uk/projects/fastqc/"(accessed on 01 March 2020).

FatiGO. Babelomics 5. Available online: http://babelomics.bioinfo.cipf.es/ (accessed on 01 May 2020).

Faustman C., Sun Q., Mancini R. & Suman S. P. (2010) Myoglobin and lipid oxidation interactions: Mechanistic bases and control. *Meat science* **86**, 86–94.

Fernández-Barroso M.A., Silió L., Rodríguez C., Palma-Granados P., López A., Caraballo C., García-Casco J.M., Sánchez-Esquiliche F., Gómez-Carballar F & Muñoz M. (2020a) Genetic Parameter Estimation and Gene Association Analyses for Meat Quality Traits in Open-Air Free-Range Iberian Pigs. *Journal Animal Breeding and Genetics*, 137: 581– 598. https://doi.org/10.1111/jbg.12498.

Fernández-Barroso M.A., Caraballo C., Silió L., Rodríguez C., Núñez Y., Sánchez-Esquiliche F., Matos G., García-Casco J.M. & Muñoz M. (2020b) Differences in loin tenderness of Iberian pigs explained through dissimilarities in their transcriptome expression profile. *Animals* **10** (9), 1715; https://doi.org/10.3390/ani10091715.

Gallagher W.M., Currid C.A. & Whelan L.C. (2005) Fibulins and cancer: friend or foe? Trends in molecular medicine **11** (7) 336–40.

Gangenahalli G.U., Gupta P., Saluja D., Verma Y.K., Kishore V., Chandra R., Sharma R.K. & Ravindranath T. (2005) Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. *Stem Cells and Development* **14** (2): 140-52.

Gianfrancesco M.A., Dehairs J., L'homme L., Herinckx G., Esser N., Jansen O., Habraken Y., Lassence C., Swinnen J.V., Rider M.H., Piette J., Paquot N. & Legrand-Poels S. (2019) Saturated fatty acids induce NLRP3 activation in human macrophages through K (+) efflux resulting from phospholipid saturation and Na, K-ATPase disruption. *Biochimica et Biophysica Acta. Molecular Cell Biology of Lipids*. 1864:1017–30. doi: 10.1016/j.bbalip.2019.04.001.

Goujon C., Moncorgé O., Bauby H., Doyle T., Ward C.C., Schaller T., Hué S., Barclay W.S., Schulz R. & Malim M.H. (2013) Human MX2 is an interferon-induced post-entry inhibitor of HIV-1 infection. *Nature*. 502 (7472): 559-562. DOI: 10.1038/nature12542.

Groeneveld E., Kovak M., & Mielenz N. (2010) Users' guide and reference manual, Version 6.0. *Institute of farm animal genetics*, Vol. VCE. Neustadt, Germany.

Groeneveld E., Kovak M., & Wang, T. (1999) Multivariate Prediction and ESTimation v. 4.1. Department of Animal Sciences, University of Illinois. EEUU.

Hanasaki K., Yamada K., Yamamoto S., Ishimoto Y., Saiga A., Ono T., Ikeda M., Notoya M., Kamitani S. & Arita H. (2002) Potent modification of low-density lipoprotein by group X secretory phospholipase A2 is linked to macrophage foam cell formation. *The Journal of Biological Chemistry*. 277 (32): 29116-29124. DOI: 10.1074/jbc.m202867200).

Hornsey, H.C. (1956). The colour of cooked cured pork. 1. Estimation of the nitric oxide–haem pigments. *Journal of the Science of Food and Agriculture* **7** (8), 534–540.

Hou X., Pu L., Wang L., Liu X., Gao H., Yan H., Zhang J., Zhang Y., Yue J., Zhang L. & Wang L. (2020). Transcriptome Analysis of Skeletal Muscle in Pigs with Divergent Residual Feed Intake Phenotypes. *DNA and Cell Biology*. 404-416. http://doi.org/10.1089/dna.2019.4878.

Hubler M.J. & Kennedy A.J. (2016) Role of lipids in the metabolism and activation of immune cells. *The Journal of Nutritional Biochemistry* **34** :1-7. doi:10.1016/j.jnutbio.2015.11.002.

Imoto A., Okada M., Okazaki T., Kitasato H., Harigae H. & Takahashi S. (2010) Metallothionein-1 isoforms and vimentin are direct PU.1 downstream target genes in leukemia cells. *The Journal Biological Chemistry* **285** (14):10300-10309. doi:10.1074/jbc.M109.095810.

Jia C., Kong X., Koltes J.E., Gou X., Yang S., Yan D., Lu S. & Wei Z. (2016) Gene Co-Expression Network Analysis Unraveling Transcriptional Regulation of High-Altitude Adaptation of Tibetan Pig. *PLoS ONE*. 11 (12): e0168161. https://doi.org/10.1371/journal.pone.0168161.

Kane M., Yadav S.S., Bitzegeio J., Kutluay S.B., Zang T., Wilson S.J., Schoggins J.W., Rice C.M., Yamashita M., Hatziioannou T. & Bieniasz P.D. (2013) MX2 is an interferon-induced inhibitor of HIV-1 infection. *Nature* **502**, 563–566.

Kemper K.E, Visscher P.M. & Goddard M.E. (2012) Genetic architecture of body size in mammals. *Genome Biology* **13** (4):244. doi: 10.1186/gb4016.

Kim D., Paggi J.M., Park C., Bennett C. & Salzberg S.L. (2019) Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology* **37** 907–915.

Kim G.D., Jeong J.Y., Hur S.J., Yang H.S., Jeon J.T., & Joo, S.T. (2010) The relationship between meat colour (CIE L* and a*), myoglobin content, and their influence on muscle fiber

characteristics and pork quality. *Korean Journal for Food Science of Animal Resources* **30**, 626–633.

King M.C., Raposo G. & Lemmon M.A. (2004) Inhibition of nuclear import and cell-cycle progression by mutated forms of the dynamin-like GTPase MxB. In Proceedings of the National Academy of Sciences of the United States of America. Jun; 101(24):8957-8962. DOI: 10.1073/pnas.0403167101.

Krämer A., Green J., Pollard J. & Tugendreich S. (2014) Causal Analysis Approaches in Ingenuity Pathway Analysis. *Bioinformatics* **30**, 523–530.

Li X., Tu L., Murphy P.G., Kadono T., Steeber D.A. & Tedder T.F. (2001) CHST1 and CHST2 sulfotransferase expression by vascular endothelial cells regulates shear-resistant leukocyte rolling via L-selectin. *Journal of Leukocyte Biology* **69** (4):565-74.

Li X.J., Yang H., Li G.X., Zhang G.H., Cheng J., Guan H. & Yang G.S. (2011) Transcriptome profile analysis of porcine adipose tissue by high-throughput sequencing. *Animal Genetics* **43** (2), 144–152.

Li X., Kim S.W., Do K.T., Ha Y.K., Lee Y.M., Yoon S.H., Kim H.B., Kim J.J., Choi B.H. & Kim K.S. (2010) Analyses of porcine public SNPs in coding-gene regions by re-sequencing and phenotypic association studies. *Molecular Biology Reports* **38** (6), 3805–3820. doi:10.1007/s11033-010-0496-1.

Li X.J., Zhou J., Liu L.Q., Qian K. & Wang C.L. (2016) Identification of genes in longissimus dorsi muscle differentially expressed between Wannanhua and Yorkshire pigs using RNA-sequencing. *Animal Genetics* **47** (3) 324-33.

Lindahl G, Enfält A.C., Seth Gv., Joseli A., Hedebro-Velander I., Andersen H.J., Braunschweig M., Andersson L. & Lundström K. (2004) A second mutant allele (V199I) and the PRKAG3 (RN) locus II. Effect of colour characteristics of pork loin. *Meat Science* **66** (3), 621–627.

Liscovitch M., Czarny M., Fiucci G. & Tang X. (2000) Phospholipase D: molecular and cell biology of a novel gene family. *The Biochemical Journal* **345** (3) 401-415.

Liu W., Baker S.S., Baker R.D., Nowak N.J. & Zhu, L. (2011) Upregulation of hemoglobin expression by oxidative stress in hepatocytes and its implication in nonalcoholic steatohepatitis. *PLoS One* 6 (9): e24363. doi: 10.1371/journal.pone.0024363.

Lopez-Bote C.J. (1998) Sustained Utilization of the Iberian Pig Breed. *Meat Science* **49** (1) 17-27.

Love M.I., Huber W. & Anders S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**, 550.

Mancini R.A. & Hunt M.C. (2005). Current research in meat colour. *Meat Science* **71**, 100–21.

Mateos M.V., Giusto N.M. & Salvador G.A. (2012) Distinctive roles of PLD signaling elicited by oxidative stress in synaptic endings from adult and aged rats. *Biochimica Biophysica acta*, 1823 (12): 2136-48. doi: 10.1016/j.bbamcr.2012.09.005.

Miron M., Woody O.Z., Marcil A., Murie C., Sladek R. & Nadon R. (2006) A Methodology for Global Validation of Microarray Experiments. *BMC Bioinformatics*, 7: 333. doi: 10.1186/1471-2105-7-333.

Muñoz M., García-Casco J.M., Caraballo C., Fernández-Barroso M.Á., Sánchez-Esquiliche F., Gómez F., Rodríguez M.C. & Silió L. (2018) Identification of Candidate Genes and Regulatory Factors Underlying Intramuscular Fat Content Through Longissimus Dorsi Transcriptome Analyses in Heavy Iberian Pigs. *Frontiers Genetics* **9**, 1–16.

Murakami M., Sato H. & Taketomi Y. (2020) Updating Phospholipase A2 *Biology Biomolecules*. 10, 1457. https://doi.org/10.3390/biom10101457.

Newcom D.W., Stalder K.J., Baas T.J., Goodwin R.N., Parrish F.C. & Wiegand B.R. (2004) Breed differences and genetic parameters of myoglobin concentration in porcine longissimus muscle. *Journal of Animal Science* **82** (8), 2264–2268.

Nishida E., & Gotoh Y. (1993) The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends in Biochemical Sciences* **8** (4): 128-131.

O'Brien P.J., Shen H., McCutcheon L.J., O'Grady M., Byrne P.J., Ferguson H.W., Mirsalimi M.S., Julian R.J., Sargeant J.M., Tremblay R.R., et al. (1992) Rapid, simple and sensitive microassay for skeletal and cardiac muscle myoglobin and hemoglobin: use in various animals indicates functional role of myohemoproteins. *Molecular and Cellular Biochemistry* **112**, 45–52 (1992).

Ollivier, L. (2009) European Pig Genetic Diversity: A. Minireview. *Animal* **3**, 915–924.

Olsson V. & Pickova J. (2005) The influence of production systems on meat quality, with emphasis on pork. *AMBIO* **34** (338-43).

Óvilo C., Benítez R., Fernández A., Núñez Y., Ayuso M., Fernández A.I., Rodríguez C., Isabel B., Rey A.I., López-Bote C. & Silió L. (2014) Longissimus Dorsi Transcriptome Analysis of Purebred and Crossbred Iberian Pigs Differing in Muscle Characteristics. BMC Genomics **31,** 15:413. doi: 10.1186/1471-2164-15-413.

Pugliese C., Bozzi R., Campodoni G., Acciaioli A., Franci O. & Gandini G. (2005) Performance of Cinta Senese pigs reared outdoors and indoors. 1. Meat and subcutaneous fat characteristics. *Meat Science* **69**, 459–464.

Puig-Oliveras A., Ballester M., Corominas J., Revilla M., Estellé J., Fernández A.I., Ramayo-Caldas Y. & Folch J.M. (2014) A Co-Association Network Analysis of the Genetic Determination of Pig Conformation, Growth and Fatness. *Plos one* **9** (12): e114862. https://doi.org/10.1371/journal.pone.0114862.

Rivera-Ferre M.G., Aguilera J.F. & Nieto R. (2005) Muscle Fractional Protein Synthesis Is Higher in Iberian than in Landrace Growing Pigs Fed Adequate or Lysine-Deficient Diets. *The journal of nutrition*, **135**, 469–478.

Ryan E.J., Marshall A.J., Magaletti D., Floyd H., Draves K.E., Olson N.E. & Clark E.A. (2002) Dendritic cell-associated lectin-1: a novel dendritic cell-associated, C-type lectin-like molecule enhances T cell secretion of IL-4. *Journal of Immunology* **169** (10): 5638-5648. DOI: 10.4049/jimmunol.169.10.5638.

Sasaki K., Tungtrakoolsub P., Morozumi T., Uenishi H., Kawahara M. & Watanabe T. (2014) A single nucleotide polymorphism of porcine MX2 gene provides antiviral activity against vesicular stomatitis virus. *Immunogenetics* **66**, 25–32.

Shen, J., Hao, Z., Wang, J., Hu, J., Liu, X., Li, S., Ke, N., Song, Y., Lu, Y., Hu, L., Qiao, L., Wu, X. & Luo, Y. (2021) Comparative Transcriptome Profile Analysis of Longissimus dorsi Muscle Tissues From Two Goat Breeds With Different Meat Production Performance Using RNA-Seq. *Frontiers Genetics* **11**: 619399. doi: 10.3389/fgene.2020.619399

Snyman H.N., Hammermueller J.D., Jagt K.J., Squires, E.J. Hayes M.A. & Lillie, B.N. Using transcriptome-wide analysis of constitutive hepatic gene expression to identify genetic defects in porcine innate immunity. 2014. In Proceedings, 10th World Congress of Genetics Applied to Livestock Production. Guelph, Ontario, (Canada).

Suman S.P. & Poulson J. (2013) Myoglobin Chemistry and Meat Colour. Annual Review of Food Science Technology **4**: 79–99.

Sun G.Y., Shelat P.B., Jensen M.B., He Y., Sun A.Y. & Simonyi, A. (2010) Phospholipases A2 and inflammatory responses in the central nervous system. *Neuromolecular Medicine* **12** (2),133-148.

Szklarczyk D., Morris J.H., Cook H., Kuhn M., Wyder S., Simonovic M., Santos A., Doncheva N.T., Roth A., Bork P., Jensen L.J., von Mering C. (2017) The STRING Database in 2017: Quality-Controlled Protein-Protein Association Networks, Made Broadly Accessible. *Nucleic Acids Research* **45 (D1)**, D362–D368.

Tang J., Faustman C. & Hoagland T.A. (2004) Krzywicki revisited: equations for spectrophotometric determination of myoglobin redox forms in aqueous meat extracts. *Journal of Food Science* **69** (9) C717–720. https://doi.org/10.1111/j.1365-2621.2004.tb09922.x.

Tartibian B., Maleki B.H. & Abbasi A. (2011). Omega-3 fatty acids supplementation attenuates inflammatory markers after eccentric exercise in untrained men. *Clinical Journal of Sport Medicine* **21** (2), 131-137.

Team, R. (2015). RStudio: Integrated development for R. Boston, MA: RStudio, Inc., Retrieved from http://www.rstudio.com

Tengvall K., Bergvall K., Olsson M., Ardesjö-Lundgren B., Farias F.H.G., Kierczak M., Hedhammar Å., Lindblad-Toh K. & Andersson G. (2020) Transcriptomes from German shepherd dogs reveal differences in immune activity between atopic dermatitis affected and control skin. *Immunogenetics* **72** (5):315-323.

TrimGalore. Available online: "http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/"(accessed on 01 March 2020).

Vadas P. & Pruzanski W. (1986) Role of secretory phospholipases A2 in the pathobiology of disease. *Laboratory Investigation* **55** (4), 391-404.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A. & Speleman F. (2002) Accurate Normalization of Real-Time Quantitative RT-PCR Data by Geometric Averaging of Multiple Internal Control Genes. *Genome Biology* **3** (7). doi: 10.1186/gb-2002-3-7-research0034.

Ventanas S., Ventanas J., Ruiz J. & Estévez M., (2005). Iberian pigs for the development of high-quality cured products. In Proceedings Recent Research Development Agricultural & Food Chemistry **6** (27–53). Research Signpost, Kerala, (India).

Vigors S., O'Doherty J.V., Bryan K. & Sweeney, T.A (2019) Comparative analysis of the transcriptome profiles of liver and muscle tissue in pigs divergent for feed efficiency. *BMC genomics* **20** (1), 461.

Vomastek T., Iwanicki M.P., Burack W.R., Tiwari D., Kumar D., Parsons J.T., Weber M.J. & Nandicoori V.K. (2008) Extracellular signal-regulated kinase 2 (ERK2) phosphorylation sites and docking domain on the nuclear pore complex protein Tpr cooperatively regulate ERK2-Tpr interaction. *Molecular Cell Biology*, **28** (22): 6954-6966.

Wang Q., Qi R., Wang J., Huang W., Wu Y., Huang X., Yang F. & Huang J. (2017) Differential expression profile of miRNAs in porcine muscle and adipose tissue during development. *Gene* **618**, 49–56.

Wei N., Wang Y., Xu R.X., Wang G.Q., Xiong Y., Yu T.Y., Yang G.S. & Pang W.J. (2015) PU.1 antisense lncRNA against its mRNA translation promotes adipogenesis in porcine preadipocytes. *Animal Genetics*. **46** (2):133-140.

Wittenberg J.B. & Wittenberg B.A. (2003) Myoglobin function reassessed. *The journal of Experimental Biology* **206**, 2011–20.

Wood J.D., Enser M., Fisher A.V., Nute G.R., Sheard P.R., Richardson R. I., Hughes S.I., Whittington F.M. (2008). Fat deposition, fatty acid composition and meat quality: A review. *Meat Science* **78** (4), 343–358.

Yang J., Yu M., Liu B., Yerle M., Fan B., Zhu M., Zhu Z. & Li K. (2004) Assignment of the porcine PLD2 gene to chromosome 12 and characterization of a SINE indel polymorphism. *Animal Genetics*. **35** (4), 352–353.

Yang Y., Zhou R., Mu Y., Hou X., Tang Z. & Li K. (2016) Genome-wide analysis of DNA methylation in obese, lean and miniature pig breeds. *Scientific Reports* **6**, 30160. https://doi.org/10.1038/srep30160.

Yu Q.P., Feng D.Y., Xiao J., Wu F., He X.J., Xia M.H., Dong T., Liu Y.H., Tan H.Z., Zou S.G., Zheng T., Ou X.H. & Zuo J.J. (2017) Studies on meat colour, myoglobin content, enzyme activities, and genes associated with oxidative potential of pigs slaughtered at different growth stages. *Asian-Australas Journal Animal Science* **30**, 12, 1739-1750.

Zappaterra M., Gioiosa S., Chillemi G., Zambonelli P., & Davoli R. (2020) Muscle Transcriptome Analysis Identifies Genes Involved in Ciliogenesis and the Molecular Cascade Associated with Intramuscular Fat Content in Large White Heavy Pigs. *PLoS ONE* 15, e0233372.

Zhang L., Michal J.J., O'Fallon J.V., Pan Z., Gaskins C.T., Reeves J.J., Busboom J.R., Zhou X., Ding B., Dodson M.V. & Jiang Z. (2012) Quantitative genomics of 30 complex phenotypes in Wagyu x Angus F_1 progeny. *Internal Journal of Biological Sciences* **8** (6), 838-58.

DISCUSIÓN GENERAL

5.1. Los caracteres de calidad y su implementación en un programa de mejora para el cerdo Ibérico

El estudio de las razas autóctonas está adquiriendo en los últimos años cada vez mayor relevancia debido al papel que juegan en la conservación de la biodiversidad, la preservación del medio rural o el mantenimiento de la tradición ganadera y cultura gastronómica. En ese sentido el cerdo Ibérico, con su volumen poblacional y alcance económico, constituye uno de los mejores ejemplos a seguir en Europa. Por tanto, no es de extrañar que el enorme desarrollo en los últimos años de las técnicas genómicas y su aplicación a las principales especies ganaderas, también esté siendo utilizado para profundizar en el conocimiento de esta raza y para la mejora de sus aspectos productivos desfavorables, pero manteniendo sus características diferenciadoras.

Aunque los programas de mejora convencionales para porcino han sido de gran utilidad, para caracteres de calidad de carne tienen una serie de limitaciones asociadas con el elevado número de registros fenotípicos necesarios, que deben ser medidos *postmortem* en animales emparentados. En los últimos tiempos ha habido un progreso importante en los métodos de análisis genómico y en los estudios de expresión génica, que han posibilitado avanzar desde el análisis individual de un gen hasta el estudio completo de su genoma y transcriptoma, así como realizar un examen exhaustivo de los mismos en toda su complejidad.

La presente tesis doctoral aborda el objetivo de incluir la información molecular obtenida de la aplicación de técnicas ómicas actuales (genómica y transcriptómica), en un programa de mejora en cerdo Ibérico centrado en caracteres de calidad novedosos, además de indagar la utilización de nuevas técnicas de fenotipado, como el NIRS, para incorporar esta información en los caracteres de calidad de carne considerados.

En primer lugar, se genotiparon los animales para un panel de SNPs localizados en genes candidatos y se realizó el análisis de asociación con los citados caracteres de calidad. En segundo lugar, se realizó un estudio de la expresión del transcriptoma en músculo a partir de animales con valores mejorantes divergentes para dos caracteres, resistencia al corte (terneza) y contenido en mioglobina (color de la carne), aplicando la secuenciación masiva del ARN (RNA-seq) para identificar genes con diferencias de

expresión en los grupos divergentes para estos dos caracteres. Estas estrategias tienen ciertas ventajas e inconvenientes. La herramienta del panel de SNPs en genes candidatos sería una opción de bajo coste y de más sencilla implementación, aunque tiene la desventaja de que necesitamos indagar previamente sobre los genes seleccionados en cuanto a su función y la presencia de polimorfismos que estén segregando en la población analizada (Li, 2013), además de la limitación de tener que elegir entre un número reducido de SNPs como objetivo de investigación. La estrategia de secuenciación de todo el transcriptoma nos ofrece la posibilidad de obtener una información más exhaustiva y completa de todos los genes que se están expresando bajo unas condiciones determinadas, así como permite estudiar las funciones, rutas metabólicas y procesos biológicos en los que estos genes con diferencias de expresión están implicados. Además, en un segundo paso, con estas secuencias podríamos identificar posibles polimorfismos localizados en regiones reguladoras en los citados genes, que podrían estar asociados a los cambios en la expresión y serían posibles candidatos como marcadores de selección genética. Con esta técnica también se pueden analizar una parte de los tránscritos expresados en el tejido estudiado y detectar isoformas no descritas hasta el momento. Sin embargo, se puede citar como inconvenientes la mayor complejidad de los análisis bioinformáticos debido a la elevada magnitud de datos que se genera, el mayor coste que implica esta metodología derivado del proceso de extracción y secuenciación de ARN o la conveniencia de validar los resultados obtenidos mediante la técnica de qPCR. Sin embargo, esta conveniencia es objeto de debate puesto que estudios recientes han descrito que la técnica RNA-seq puede ser un método suficientemente robusto y, por tanto, no siempre debe ser necesaria esta validación (Coenye, 2021).

A continuación, se realiza una discusión de cada uno de los experimentos realizados.

5.2. La estimación de parámetros genéticos y análisis de asociación para caracteres de calidad de carne

En este trabajo se ha pretendido explorar la inclusión de los caracteres de calidad en un programa de mejora genética de cerdos Ibéricos de montanera. Para ello, previamente, se ha llevado a cabo la caracterización fenotípica y genética de la

población objeto de estudio. Las estimas de heredabilidades y correlaciones genéticas obtenidas presentan valores diversos cuando se comparan con otros trabajos, tanto en Ibérico como con otras razas, de ahí la relevancia que tiene realizar estas estimaciones en la población concreta donde se va a implementar el programa, especialmente si se trata de cerdos Ibéricos de montanera.

Los parámetros genéticos estimados mostraron valores moderados de heredabilidad para el contenido en mioglobina, las pérdidas de agua por descongelado y por fuerza centrífuga (0.15 a 0.19), valores un poco más elevados para la resistencia al corte y el valor de colorímetro rojo (a*) (0.25 ambos) y una heredabilidad moderada-alta para las pérdidas de agua por cocinado (0.43), por lo que en un programa de mejora que incluyese estos tres últimos caracteres se obtendría una evidente respuesta positiva a la selección. Además, las correlaciones genéticas de las tres medidas de pérdidas de agua entre sí (0.93 a 0.96) y a su vez, éstas con la resistencia al corte (0.71 a 0.91) fueron positivas y elevadas, siendo por tanto favorable para la selección, ya que ofrece la posibilidad de utilizar como criterio cualquiera de los tres tipos de pérdidas de agua sin perjudicar la resistencia al corte. En este sentido, en el presente trabajo se ha observado que carnes con menores pérdidas de agua también resultan ser más tiernas. Como se ha mencionado previamente a lo largo de la tesis, el color rojo de la carne es uno de los atributos más apreciados por el consumidor y ha sido asociado con sistemas productivos en extensivo (Pugliese et al., 2005), por lo que resulta de interés incluirlo en un programa al menos con el fin de evitar un posible deterioro del color de la carne por respuestas no deseadas a la selección. De hecho, el atributo de color más heredable fue el rojo (a*) y además se obtuvo una correlación genética positiva y elevada entre a* y el contenido en mioglobina (0.94), por lo que un programa con a* como criterio, que es más fácil de medir, podría mejorar el contenido de mioglobina en carne como objetivo de selección. Como resultado no del todo deseable, se halló una correlación positiva y elevada entre las pérdidas de agua y el valor de colorímetro luminosidad (L*), resultado que refuerza la teoría de que la capacidad de retención de agua se ve reducida en carnes más pálidas, como las citadas carnes PSE. Finalmente, las correlaciones genéticas obtenidas también mostraron que los valores de color a* y b* están relacionados con las pérdidas de agua, de forma positiva y en un rango moderado, por lo que en futuros estudios se debería

comprobar este posible efecto negativo de la mejora del color rojo de la carne sobre las pérdidas de agua.

Los análisis de estimación de parámetros llevados a cabo en muestras de lomo curado mostraron que las heredabilidades en los dos caracteres de textura estudiados, resistencia al corte y fuerza máxima de compresión (SFC y F0, respectivamente), eran cercanas a cero. Este resultado se explica porque gran parte de la varianza se puede atribuir al efecto ambiental de los lotes de montanera y sacrificio. Además, el proceso de curación implica una serie de fases como salado, marinado, secado y curado, cuyo ajuste en el modelo lineal de análisis es muy complejo, y que explicarían los altos valores observados en la varianza residual. Por otro lado, la correlación genética entre los dos caracteres fue elevada (0.87), aunque con un error estándar también alto. Estos resultados indicarían la dificultad de trabajar con caracteres medidos en producto curado para poder ser incluidos en un esquema de selección genética.

La segunda parte del primer experimento está orientada hacia la búsqueda, mediante un panel de SNPs, de polimorfismos en genes candidatos con posibles efectos en los caracteres de calidad. Como se ha indicado, esta estrategia es de bajo coste y sencilla de aplicar, si bien las peculiaridades de las razas autóctonas respecto al tamaño poblacional y su adaptación a entornos ambientales concretos, pueden ser motivo de diferencias en la variabilidad y presencia de polimorfismos distintos. Utilizando los datos de secuenciación del genoma completo de 15 individuos Ibéricos generados en un estudio previo del grupo de investigación (Muñoz et al., 2020) se detectaron polimorfismos segregando en genes relacionados con calidad de la carne, que se utilizaron, junto con las reseñas bibliográficas, para el diseño del panel de SNPs. Con su aplicación en nuestra población porcina, en la presente tesis se han descrito por primera vez en cerdo Ibérico asociaciones de observaciones fenotípicas en varios caracteres de calidad con un conjunto de SNPs localizados en genes candidatos, concretamente fueron mapeados en los genes *PRKAG3, CAPN1, CAPNS1, CAPNS2, CASP3, CASP9, CTSL, MYOD* y *MYH3.*

Dos SNPs localizados en el gen *PRKAG3 (rs319678464G > C* y *rs330427832C > T)* tienen una asociación significativa sobre los caracteres de pérdidas de agua y parámetros de color rojo (a*) y amarillo (b*), en los cuales los alelos G y C,

respectivamente, estaban asociados a menores pérdidas de agua y, a su vez, con carne menos rojiza. Los resultados obtenidos están en línea con lo encontrado por otros estudios en porcinos de raza magra que han descrito SNPs en este gen con efectos para las pérdidas de agua, el pH, el valor de L* y a*.

El gen *CAPN1* codifica para varias isoformas de la calpaína, implicada en el proceso de proteólisis y asociada con la maduración de la carne en diversas especies animales, entre ellas el cerdo (Koohmaraie & Geesink, 2006). En nuestro estudio de asociación se encontraron efectos significativos del SNP *CAPN1_ rs81358667G > A* para la resistencia al corte y las pérdidas de agua por fuerza centrífuga, y a la vez se observó un efecto relevante del *CAPN1_ rs81358636G > A* en resistencia al corte. Por ello, hemos considerado al primero de ellos como un buen candidato para seleccionar cerdos de montanera con carnes más tiernas.

Cabe destacar la importancia que han tenido los resultados obtenidos para dos SNPs mapeados en el gen *CASP9 (rs324307617C > T* y *rs346077419G > A),* ya que es la primera vez que se han observado en porcino asociaciones significativas de polimorfismos para el contenido en mioglobina en músculo. También se observó una asociación en la misma dirección del SNP *CASP9_rs346188168G > A* con el color rojo a*, aunque esta no fue significativa. Además, puesto que las caspasas participan como proteasas en el proceso de maduración y proteólisis de la carne, en nuestro estudio hemos encontrado efectos significativos favorables del *CASP3_rs319658214G > T* para la terneza y las pérdidas de agua, en los cuales el alelo G estuvo asociado a mayor terneza y menores pérdidas de agua, respectivamente. Por lo tanto, podemos asociar un efecto de este polimorfismo con una mayor terneza de la carne y considerarlo adecuado para ser propuesto como gen candidato.

Otro aspecto relevante de los trabajos realizados en este capítulo ha sido la detección por primera vez en porcino de asociaciones significativas de algunos de los SNPs localizados en los genes *MYH3* y *CTSL* con las pérdidas de agua por cocinado y con las pérdidas de agua y el color L*, respectivamente; junto a los ya citados en *CASP3 y CASP9*.

Los análisis de asociación realizados en caracteres de textura medidos en lomo curado, en consonancia con las estimaciones previas de heredabilidades, no resultaron en ningún caso en asociaciones significativas, lo cual refuerza la idea sobre la dificultad de poder ser incluidos en programas de mejora.

5.3. Determinación de caracteres de calidad mediante tecnología NIRS

La caracterización fenotípica de estos caracteres de calidad propuestos para un programa de mejora en cerdo Ibérico se suele llevar a cabo con técnicas laboriosas, mediante métodos físico-químicos de referencia que consumen una cantidad considerable de tiempo y además no suelen estar adaptadas para un análisis en tiempo real. Por ello, el segundo experimento tuvo como objetivo calibrar y evaluar la precisión de la tecnología NIRS para poder determinar simultáneamente el conjunto de caracteres de calidad, como procedimiento alternativo a los métodos de referencia del laboratorio. De este modo se podría medir de forma rápida, limpia y de una sola vez el elevado número de registros que se necesitan para incluir estos caracteres en un esquema de selección genética.

El éxito del calibrado de la técnica NIRS para la determinación del contenido en proteína, humedad o grasa intramuscular en muestras de carne de cerdo Ibérico, está acreditado (Solís et al., 2001; Zamora-Rojas et al., 2012; Cáceres-Nevado et al., 2019). Con este antecedente, se llevó a cabo el estudio para la calibración un equipo NIRS con alrededor de 300 muestras procedentes de músculo longissimus dorsi. El objetivo fue determinar simultáneamente 10 caracteres de calidad de carne (L*, a*, b*, contenido en mioglobina, pérdidas de agua por fuerza centrífuga y los caracteres de textura de resistencia al corte, dureza, cohesividad, elasticidad y masticabilidad) comparando además dos tipos de presentación de muestra, enteras y trituradas. Un resultado adecuado de calibración de NIRS en muestras enteras evitaría destruir una pieza de elevado valor económico para la industria.

Para valorar la calibración del NIRS en cada carácter de calidad, se evaluaron los mejores modelos en función del coeficiente de determinación más alto (R^2) y el error más bajo (RMSE). La desviación de predicción residual (RPD) se usó para evaluar la bondad de ajuste y la precisión del modelo. Además, la ratio del rango de error (RER) se

utilizó para conocer la idoneidad de los modelos de predicción para clasificar o cuantificar las muestras. Los valores estadísticos obtenidos mostraron la dificultad para obtener una cuantificación simultánea de los 10 caracteres de calidad analizados mediante NIRS, tanto en muestras intactas como en trituradas.

Las muestras tomadas de músculo constituyen, en sí mismas, muestras heterogéneas, con diferentes características físico-químicas debidas a un conjunto de factores como la organización fibrilar, la precipitación de proteínas en el sarcómero, la longitud del sarcómero, el pH, la humedad, la grasa intramuscular o las propiedades de reflectancia superficial macroscópicas (Cozzolino et al., 2000). Esta heterogeneidad explica la homogeneización de la muestra que se realiza habitualmente mediante triturado. También podría ser responsable del peor rendimiento de los modelos de calibración del NIRS para algunos de los caracteres de calidad analizados, incluso en las muestras trituradas. Aun así, es interesante resaltar que algunos de los resultados obtenidos en este ensayo han mostrado mejores valores de predicción en muestras enteras que trituradas, como por ejemplo para L*, a*, b*y dureza.

Concretando los resultados, en este experimento se ha conseguido calibrar el NIRS para la cuantificación de resistencia al corte en muestras trituradas, hallazgo muy prometedor ya que la técnica de referencia que se usa actualmente para cuantificar la resistencia al corte (Warner-Bratzler), es laboriosa y requiere de múltiples réplicas (al menos entre 6 y 8) debido a la elevada variación existente dentro de la misma muestra. También se ha podido calibrar NIRS para una clasificación de las muestras en tres categorías (alto, medio y bajo) para la cohesividad (textura) en muestras trituradas y para el color L*, a*, b* y la dureza (textura) en muestras enteras. En definitiva, aunque el resultado final obtenido no haya sido totalmente el deseado, es importante mencionar que es la primera vez que se lleva a cabo un estudio de calibración de NIRS para medir simultáneamente, en muestras trituradas y enteras, el contenido en mioglobina, los parámetros de color, las pérdidas de agua y los diferentes parámetros de textura, además de ser la primera vez que se aplica NIRS para medir en porcino el contenido en mioglobina y la pérdida de agua por fuerza centrífuga.

Como reflexión final en este capítulo, tan sólo indicar que se deberían tener en cuenta algunas consideraciones para mejorar la precisión de la técnica NIRS en futuras

mediciones de este tipo de caracteres. En primer lugar, cuando existe poca varianza en la distribución de los valores de las muestras medidos mediante la técnica de referencia y, por tanto, la distribución es más estrecha, los valores para R^2 y RPD no pueden ser lo sufrientemente elevados (Pérez-Marín et al., 2004). Por lo tanto sería necesario aumentar el tamaño muestral, con una selección de muestras con mayores probabilidades de divergencia, con el fin de incrementar esta varianza y hacer más ancha la distribución. Por último, para intentar mejorar la predicción de los caracteres de color, en futuros estudios de calibración sería aconsejable añadir al ensayo la parte del espectro visible (400 – 750 nm) (Ripoll et al., 2008; Monteiro-Balage et al., 2015).

5.4. La terneza y el contenido en mioglobina del lomo. Análisis de las diferencias de expresión en el perfil del transcriptoma

La razón por la que se ha seleccionado para el estudio del transcriptoma caracteres de calidad como terneza (medida como resistencia al corte con texturómetro) y contenido en mioglobina, está justificada, como se ha descrito anteriormente, en su importancia para el consumo de carne en fresco, puesto que son dos de los atributos de calidad (terneza y el color rojo cereza brillante) más apreciados por el consumidor. Además, el moderado valor de sus heredabilidades convierte a estos caracteres en candidatos aptos para la búsqueda de genes candidatos. Finalmente, el novedoso análisis de transcriptoma en cerdos en general, y en Ibéricos en particular, para terneza y mioglobina, también fue determinante a la hora de su elección. La estrategia utilizada en estos experimentos, con el análisis RNA-seq de transcriptoma completo en muestras de lomo, ha permitido identificar, caracterizar y cuantificar un conjunto de genes y tránscritos expresados diferencialmente en función de los caracteres de terneza y contenido en mioglobina. Sin embargo, en ninguno de los dos casos se han detectado como genes diferencialmente expresados los incluidos en el análisis de asociación llevado a cabo en el primer experimento.

El análisis de expresión diferencial del transcriptoma de individuos divergentes en su valor mejorante para el carácter de terneza, reveló 200 genes diferencialmente expresados (DEGs) en el lomo. El análisis funcional llevado a cabo mostró que estos DEGs estaban sobrerrepresentados en procesos relacionados con la proteólisis, el desarrollo

del tejido muscular y los metabolismos lipídico y del colágeno. El hecho de identificar DEGs implicados en procesos de proteólisis ha sido un hallazgo relevante, ya que, como se ha mencionado en el primer capítulo, la proteólisis juega un papel fundamental en la maduración de la carne. En este proceso, las catepsinas degradan la fuerte unión de actomiosina, lo que produce el ensanchamiento de los sarcómeros. Resulta interesante que en nuestro estudio se ha observado en el grupo de cerdos con carne más tierna una mayor expresión del gen que codifica para la catepsina C (*CTSC*). El análisis funcional confirmó que este gen está implicado en rutas relacionadas con la regulación de la proteólisis, por lo que se puede asociar una mayor expresión del gen *CTSC* con una mayor actividad proteolítica, dando lugar a una carne más tierna. Otros DEGs que codifican proteínas implicadas en procesos *post-mortem* relacionados con la proteólisis fueron *RHOD, MYH8, ACTC1, GADD45B, CASQ2, CHRNA9* y *ANKRD1.*

El metabolismo lipídico ha sido otro de los procesos biológicos relevantes vinculado con la expresión diferencial. El contenido en grasa intramuscular es uno de los principales determinantes de la terneza de la carne y se ha descrito una relación positiva entre ambos caracteres (Wood, 1990), además la correlación genética entre ellos sugiere que pueden compartir una base genética común (Sellier, 1998; Lee et al., 2015). De este modo, se identificaron genes implicados en procesos relacionados con metabolismo lipídico y sobreexpresados en el grupo de cerdos con carne más tierna, como *FABP3* y *PPARGC1A*.

Asimismo, se ha descrito que existe un moderado antagonismo entre el contenido en grasa intramuscular y el desarrollo muscular en cerdos (Hocquette et al., 2010). Por lo tanto, la relación inversa entre estos caracteres implicaría que una selección basada en un mayor desarrollo muscular y producción de carne tendría efectos negativos en la terneza. De este modo, el análisis del transcriptoma llevado a cabo reveló DEGs en el grupo de animales con carne más dura que estaban implicados en procesos de desarrollo muscular y crecimiento (*DMD, FOS* y *MSTN*) y contenido en colágeno *(COL14A1),* que también ha sido relacionado por otros autores por su contribución a la dureza de la carne (Torrescano et al., 2003; Purslow, 2005). Los resultados obtenidos para el gen *MSTN* generan cierta contradicción, ya que codifica para la miostatina, proteína asociada con la inhibición de la miogénesis, aunque en el presente estudio se

observó una mayor expresión de este gen en el grupo de animales con mayor dureza de carne. Además, en este experimento se identificaron una serie de reguladores de la expresión como son IGF1, VGLL3 y PPARG. En nuestro estudio IGF1 parece estar regulando la expresión de genes asociados con desarrollo muscular y que están sobreexpresados en cerdos con carne más dura (*FOS* y *COL1A1*), a su vez, inhibe la expresión de genes relacionados con metabolismo lipídico (*CEBPD* y *PSMB8*). VGLL3 regula la expresión de genes involucrados en procesos del metabolismo del colágeno y desarrollo celular (*COL12A1* y *GADD45B*). Mientras que PPARG, ha sido relacionado como un regulador de procesos del metabolismo lipídico (Zimmerman et al., 2002), además en nuestro estudio ha sido identificado como activador de la expresión de genes implicados en la adipogénesis (*FABP3* and *PPARGC1A*).

El análisis de expresión diferencial del transcriptoma de individuos extremos en su valor mejorante para el contenido en mioglobina, reveló un total de 57 DEGs. El primer resultado esperado sería que entre ellos estuviera el gen que codifica para la mioglobina, sin embargo, el gen *MB* no está anotado en la versión del genoma porcino utilizada en el análisis de los datos de secuenciación. El análisis de expresión diferencial sí identificó al gen que codifica para la hemoglobina subunidad 1 (*HBA1*), sobreexpresada en el grupo de mayor contenido en mioglobina, relacionada con el transporte de oxígeno y protección frente a stress oxidativo del músculo. El análisis funcional mostró que *HBA1* tiene un papel relevante en los procesos metabólicos de las especies reactivas de oxígeno (ROS).

Otros dos genes que codifican para fosfolipasas, *PLAG2G10* y *PLD2*, también mostraron sobreexpresión en el grupo de mayor contenido en mioglobina, ambos relacionados con metabolismo lipídico. Además, se ha descrito que *PLA2G10* está asociado con funciones inmunológicas y respuestas antiinflamatorias (Murakami et al., 2020). El gen *ECH1*, reprimido en animales con mayor contenido en mioglobina, también está asociado al metabolismo lipídico, si bien este gen codifica una enzima asociada con la β-oxidación lipídica que se produce en la mitocondria, distinta de la oxidación en los lípidos de membranas.

Los procesos de oxidación-reducción están estrechamente relacionados con los estados químicos en los que la mioglobina se encuentra en la carne (deoximioglobina,

oximioglobina, metamioglobina y carboximioglobina). Los agentes prooxidantes como los ROS, además de promover la rancidez, pueden afectar a la estabilidad del color rojo de la carne debido a la oxidación de la mioglobina. En este sentido, Li et al. (2016a) describieron genes diferencialmente expresados implicados en el metabolismo de la oxidación lipídica, los cuales podrían estar promoviendo a su vez la oxidación de la mioglobina, pudiendo acentuar el color marrón no deseable de la carne, ya citado. Por ello, estrategias centradas en reducir la oxidación lipídica podrían frenar el enranciamiento y mejorar la estabilidad del color. Por ejemplo, se ha descrito que antioxidantes como la Vitamina C y E tienen un rol protector, pudendo mejorar el color de la carne y la estabilidad lipídica. En esta dirección, el análisis funcional realizado con IPA identificó a los genes *PLA2G10* y *PLD2* con mayor expresión en animales con mayor contenido en mioglobina, como implicados en la ruta *Acción antioxidante de la vitamina C.* Los resultados obtenidos, de manera global, apuntan a que músculos que pueden estar sufriendo más procesos oxidativos, parecen mostrar mayor contenido en mioglobina, debido a un mecanismo de protección de ésta.

El análisis funcional también reveló que una serie de genes sobreexpresados en el grupo de mayor contenido en mioglobina estaban implicados en mecanismos del sistema inmune (*MX2*, *CD209*, *SLA1*, *SLA7*, *LGALS3* y *LGALS9*) y respuesta inflamatoria (*CHST1*). El gen *CD209* codifica para una proteína asociada con la regulación de la proliferación de células T. La disminución de este marcador en sangre se ha relacionado con una menor inflamación, por lo que es considerado como un marcador de la misma (Tengvall et al 2020). Por último, se identificaron dos genes sobreexpresados en el grupo de cerdos con mayor contenido en mioglobina implicados en procesos de desarrollo del tejido conectivo (*EFEMP1*) y el metabolismo del colágeno (*COL12A1*).

Para finalizar, el análisis funcional predijo una serie de factores de regulación Entre ellos, algunos fueron reconocidos como *master regulators,* es decir, moléculas que se encuentran en la parte superior de una jerarquía reguladora, expresándose al inicio de un linaje de desarrollo y participando en la regulación de múltiples genes. Dos de los más importantes en el análisis del transcriptoma del músculo fueron MAPK1 y SPI1. MAPK1 ha sido reconocido como un regulador transcripcional involucrado en la diferenciación de miocitos en cerdo. En nuestro estudio SPI1 parece estar implicado en

mecanismos de regulación de los genes *CD209* y *FES*. Además, este mismo *master regulator* ha sido relacionado con la regulación de la expresión de diversos genes que participan en el metabolismo inmune (Gangenahalli et al., 2005).

Por lo tanto, en este experimento se han observado una serie de marcadores relacionados con la inflamación y con la oxidación lipídica que parecen estar relacionados con un mayor contenido en mioglobina. Los cerdos con mayor contenido en mioglobina parecen tener activados mecanismos de oxidación lipídica. Se puede hipotetizar que este incremento en la oxidación podría aumentar el contenido en hemoglobina y mioglobina en músculo, tal y como hemos sugerido proporcionando un efecto protector contra el estrés oxidativo. Aun así, se deberán realizar una serie de estudios funcionales que puedan confirmar esta hipótesis. En cualquier caso, los resultados obtenidos permiten proponer los genes *HBA1*, *CD209*, *PLA2G10* y *PLD2* como candidatos para el contenido en mioglobina y como consecuencia, para el color rojo.

5.5. Futuras aplicaciones de los resultados de la tesis. Reflexión final

En la presente tesis se ha abordado la utilización de nuevas técnicas ómicas y de registro fenotípico en programas de mejora genética en cerdo Ibérico que incluyan, como objetivo relevante, la calidad de la carne. Los resultados obtenidos se pueden encuadrar en dos tipos respecto a su aplicación inmediata en un programa. Por un lado, están aquellos que pueden tener una implementación más cercana en el tiempo, por otro, aquellos que están en una etapa más básica de estudio. En el primer grupo se encuentran, además de las estimas de componentes de varianza imprescindibles en cualquier programa, los resultados del análisis de asociación del panel de 32 marcadores con los caracteres de calidad, que ha permitido identificar una serie de polimorfismos, *PRKAG3 (rs319678464G > C* y *rs330427832C > T)* y *CAPN1_ rs81358667G > A,* con efectos sobre las pérdidas de agua y la terneza, respectivamente, que están siendo actualmente validados por el grupo de investigación para ser utilizados a corto plazo como criterio de selección de verracos, tanto de la propia explotación como procedentes de otras ganaderías. Para ello, es necesario confirmar con un diseño *ad hoc* que los alelos favorables para los caracteres mencionados no perjudiquen para otros importantes económicamente, como son el rendimiento de las piezas nobles o el porcentaje de grasa intramuscular. Además, al no saber si los polimorfismos son realmente las mutaciones

causales, hay que asegurarse de que esta asociación no se pierde con el paso de las generaciones, debido a un cambio en el desequilibrio de ligamiento entre el marcador y la potencial mutación causal. Pese a estas cautelas, los resultados obtenidos son bastante esperanzadores, y posiblemente en los próximos años la información de estos marcadores será utilizada como criterio de selección adicional en la población de cerdos Ibéricos analizada.

La comprobación de si estos polimorfismos son las mutaciones causales subyacentes al carácter no era objeto de la presente tesis. No obstante, en el futuro se deberían llevar a cabo estudios funcionales que permitan aceptar o descartar esta posibilidad. Además, al no poder determinar estos polimorfismos como mutaciones causales, hay que validar previamente estos resultados en otras poblaciones de cerdo Ibérico para proceder a su aplicación en ellas. La conocida heterogeneidad fenotípica de esta raza podría tener una correspondencia genética donde los polimorfismos analizados pudieran tener diferente grado de desequilibrio de ligamiento con las mutaciones causales en las distintas poblaciones.

Respecto al segundo experimento con NIRS, aunque no haya sido alcanzado el objetivo marcado de poder analizar varios caracteres de calidad simultáneamente en muestras enteras de carne para evitar una destrucción de la misma, sí se puede vislumbrar una aplicación cercana de esta técnica en el laboratorio. Desde el punto de vista de un programa de mejora, los resultados obtenidos con muestras trituradas en la determinación de la resistencia al corte apuntan a una utilización adecuada del NIRS para cuantificar este carácter, pudiendo sustituir en un futuro próximo a la técnica de Warner-Bratzler con texturómetro. Desde un punto de vista más general, los resultados observados en muestras intactas indican que la carne podría ser clasificada por las industrias de procesamiento de canales o de elaboración de productos en tres categorías para los caracteres de dureza y de color de manera conjunta, si bien para la clasificación sólo de los parámetros de color la medición con la técnica de referencia (colorímetro Minolta) es rápida y poco laboriosa.

Los resultados de los experimentos de transcriptoma son los que están en una etapa de conocimiento más básica, pero no por ello son menos interesantes. Este experimento nos permite proponer una serie de genes candidatos para resistencia al

corte (terneza) como son *ACTC1, ANKRD1, CHRNA9, CTSC*, *COL14A1, DMD, FABP3, FOS, IGF1, PPARG, PPARGC1A, RHOD*, y *VGLL3*, y para el contenido en mioglobina como *CD209, CHST1, ECH1, HBA1, LGALS3, LGALS9, MAPK1*, *MX2, PLA2G10, PLD2 SLA1*, *SLA7* y *SPI1* que pueden ser estudiados en mayor profundidad y, en su caso, considerados para poder seleccionar los reproductores en un futuro. Además, hemos obtenido una información muy valiosa en cuanto a las rutas biológicas implicadas en la variabilidad de estos caracteres, con una relativa sorpresa respecto al posible papel del contenido en mioglobina en el metabolismo del cerdo. Nuevamente desde un punto de vista aplicado a programas de mejora, el siguiente paso sería la búsqueda de polimorfismos localizados en estos genes que tengan las máximas diferencias alélicas en los grupos divergentes para, seguidamente, realizar los correspondientes análisis de asociación con ellos y con los demás caracteres de calidad, y así finalizar en el diseño de potenciales paneles de SNPs para la selección de los reproductores.

Los trabajos de la presente Tesis Doctoral constituyen un ejemplo de la aplicación de las técnicas ómicas en un programa de mejora de cerdo Ibérico para caracteres de calidad de carne, con el añadido de trabajar con una población sujeta a los fuertes condicionantes ambientales del manejo tradicional de la preparación de los animales para la montanera y el posterior aprovechamiento de los recursos naturales.

CONCLUSIONES/ CONCLUSIONS

1. Los valores estimados de heredabilidad y correlaciones genéticas para las pérdidas de agua por descongelado, cocinado y fuerza centrífuga, la resistencia al corte en carne cocinada, el contenido en mioglobina y el color rojo (a*), sugieren que los caracteres son apropiados para ser incluidos en un programa de mejora.

2. Dos SNPs mapeados en el gen *PRKAG3* (*rs319678464G > C* y *rs330427832C > T*), uno en el gen *MYH3* (*rs81437544T > C*), uno en *CASP3* (*rs319658214G > T*) y dos en *CTSL* (*rs332171512A > G* y *rs321623592A > T*) mostraron los mayores efectos para las pérdidas de agua. Un SNP mapeado en el gen *CAPN1* (*rs81358667G > A*) y otro en *CASP3* (*rs319658214G > T*) mostraron los mayores efectos para la resistencia al corte. Los SNPs identificados en el gen *PRKAG3* mostraron los mayores efectos en los caracteres de color. Todos estos efectos han sido descritos por primera vez en Ibérico.

3. Es la primera vez que se han descrito en carne de porcino efectos significativos de SNPs para el contenido en mioglobina (*CASP9_rs324307617C > T* y *CASP9_rs346077419G > A*).

4. Los resultados han mostrado la dificultad del NIRS para cuantificar simultáneamente diez caracteres de calidad, con una sola lectura y usando todo el espectro del infrarrojo cercano, en muestras de lomo procedentes de cerdos Ibéricos de montanera.

5. La técnica NIRS puede cuantificar el carácter resistencia al corte en muestras trituradas. Además, se puede llevar a cabo una clasificación aproximada en tres grupos (alto, medio, bajo) para el carácter cohesividad en muestras trituradas y los parámetros de color (L*, a*, b*) y dureza en muestras enteras.

6. El análisis del transcriptoma del músculo longissimus para terneza mostró 200 genes diferencialmente expresados (DEGs) y 245 isoformas no descritas. Los análisis funcionales revelaron que los DEGs estaban involucrados en procesos biológicos, rutas y redes génicas relevantes, potencialmente relacionados con terneza. Las funciones más representativas asociadas a estos caracteres fueron proteólisis, desarrollo muscular, metabolismo lipídico y metabolismo del colágeno.

7. En general, los genes que codifican proteínas involucradas en proteólisis y en la transformación del músculo en carne (*ANKRD1*, *CASQ2*, *CHRNA9*, *CTSC* y *RHOD*) y en metabolismo lipídico (*FABP3* y *PPARGC1A*) están sobreexpresados en el grupo de carne más tierna. Mientras que los genes que codifican proteínas relacionadas con miogénesis y desarrollo muscular (*FOS* y *DMD*) y metabolismo del colágeno (*COL14A1*), estuvieron sobreexpresados en el grupo de carne más dura. También se identificaron una serie de factores de regulación transcripcional relevantes para desarrollo muscular (IGF1), metabolismo del colágeno y desarrollo celular (VGLL3) y metabolismo lipídico (PPARG).

8. El análisis del transcriptoma del músculo longissimus para el contenido en mioglobina reveló 57 DEGs. El análisis funcional mostró DEGs que codifican para proteínas involucradas en procesos relacionados con transporte de oxígeno y oxidación (*HBA1*), inflamación (*CHST1, CD209* y *PLA2G10*), metabolismo lipídico (*PLA2G10*, *PLD2* y *ECH1*) y sistema inmune (*MX2*, *CD209*, *SLA1*, *SLA7*, *LGALS3* y *LGALS9*). Además, se identificaron una serie de *master regulators* con funciones relevantes para el sistema inmune y el metabolismo lipídico (SPI1) y la diferenciación de miocitos (MAPK1).

9. Los resultados sugieren que las proteínas proinflamatorias y las implicadas en oxidación lipídica podrían promover la expresión del gen *HBA1* y el aumento del contenido en mioglobina, ya que ambos parecen tener un efecto protector contra el estrés oxidativo.

1. The estimated heritability and genetic correlations values for thawing, cooking and centrifuge force water losses, shear force on cooked meat, myoglobin content and redness Minolta (a*) suggest that these traits are suitable to be included in a breeding scheme.

2. Two SNPs mapping on *PRKAG3* (*rs319678464G > C* and *rs330427832C > T*), one on *MYH3* (*rs81437544T > C*), one on *CASP3* (*rs319658214G > T*) and two on *CTSL* (*rs332171512A > G* and *rs321623592A > T*) showed the larger effects on water losses. One SNP mapping on *CAPN1* (*rs81358667G > A*) and one in *CASP3* (*rs319658214G > T*) showed the highest effects on shear force. The SNPs mapping on *PRKAG3* showed the highest effects on colour. They have been all described at the first time in the Iberian breed.

3. This is the first time that SNPs with significant effects on myoglobin content has been described in porcine meat (*CASP9_rs324307617C > T* and *CASP9_rs346077419G > A*).

4. The results revealed the difficulty of NIRS to simultaneously quantify ten meat quality traits, in a single reading, using the full spectra range in samples of longissimus dorsi from Iberian pigs fattened in an open-air free-range system.

5. NIRS seems to allow the quantification of shear force in minced samples, while a rough classification is possible both of cohesiveness in minced samples and of colour parameters (L*, a*, b*) and hardness in intact samples.

6. The transcriptome analysis for tenderness reported 200 differentially expressed annotated genes (DEGs) and 245 newly predicted isoforms on longissimus dorsi muscle. The functional analyses revealed that DEGs are involved in relevant biological processes, canonical pathways and networks potentially related with tenderness. The most representative functions associated with this trait are proteolysis, skeletal muscle development, lipid metabolism and collagen metabolism.

7. In general, genes encoding for proteins involved in proteolysis and conversion of muscle to meat (*ANKRD1*, *CASQ2*, *CHRNA9*, *CTSC* and *RHOD*) and genes involved in lipid metabolism (*FABP3* and *PPARGC1A*) are overexpressed in the Tender group. While genes encoding for proteins enhancing myogenesis and muscle development (*FOS* and *DMD*) and collagen metabolisms (*COL14A1*) are overexpressed in the Tough group. A set of transcriptional regulatory factors were identified with relevant role in muscle growth (IGF1), collagen metabolism and cellular development (VGLL3) and lipid metabolism (PPARG).

8. The transcriptome analysis for myoglobin content reported 57 DEGs on the longissimus dorsi muscle. The functional analyses revealed that DEGs codify for proteins involved in processes related with oxygen transport and oxidation (*HBA1*), inflammation (*CHST1, CD209* and *PLA2G10*), lipid metabolism (*PLA2G10*, *PLD2* and *ECH1*) and immune defence (*MX2*, *CD209*, *SLA1*, *SLA7*, *LGALS3* and *LGALS9*). Moreover, a set of master regulators with pivotal role on immune system and lipid metabolism (SPI1) and differentiation of porcine myocytes (MAPK1) were identified.

9. The results suggest that pro-inflammatory proteins and those involved in lipid oxidation could be inducing the expression of *HBA1* gene and the myoglobin content since both have a protective effect against oxidative stress.

REFERENCIAS

Adzitey, F., Nurul, H. 2011. Pale soft exudative (PSE) and dark firm dry (DFD) meats: causes and measures to reduce these incidences – a mini review. International Food Research Journal. 18, 11-20.

AECERIBER. 1998. Propuesta de índices genéticos de selección para el cerdo Ibérico. Sólo Cerdo Ibérico, número 1, 15-25.

Alberti, P., Panea, B., Ripoll, G., Sañudo, C., Olleta, J.L., Hegueruela, I., Campo, M.M., Serra, X. 2005. Medición del color. In Estandarización de las metodologías para evaluar la calidad del producto (animal vivo, canal, carne y grasa) en los rumiantes. Serie Ganadería N.º 3. (pp. 216-225). Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid (Spain).

Albuquerque, A., Óvilo, C., Núñez, Y., Benítez, R., López-García, A., García, F., Félix, M.d.R., Laranjo, M., Charneca, R., Martins, J.M. 2020. Comparative Transcriptomic Analysis of Subcutaneous Adipose Tissue from Local Pig Breeds. Genes, 11, 422. https://doi.org/10.3390/genes11040422.

Albuquerque, A., Óvilo, C., Núñez, Y., Benítez, R., López-García, A., García, F., Félix, M.d.R., Laranjo, M., Charneca, R., Martins, J.M. 2021. Transcriptomic Profiling of Skeletal Muscle Reveals Candidate Genes Influencing Muscle Growth and Associated Lipid Composition in Portuguese Local Pig Breeds. Animals, 11, 1423. https://doi.org/10.3390/ani11051423.

Alves, E., Óvilo, C., Rodríguez, M. C., Silió, L. 2003. Mitochondrial DNA sequence variation and phylogenetic relationships among Iberian pigs and other domestic and wild pig populations. Animal Genetics, 34 (5), 319–324.

Alves, E., Fernández, A., Óvilo, C., De Pedro E., Rodrigáñez, C., Silió L. 2005. Influencia de genes mitocondriales sobre el contenido de grasa intramuscular en cerdos Ibéricos. Información Técnica Económica Agraria. 26 (I), 9-11.

Alves, E., Fernández, A.I., Rodríguez, M.C., Óvilo, C. y Silió, L. 2008. Tres pesquisas sobre el origen del Manchado de Jabugo. En XIV Reunión Nacional de Mejora Genética Animal Diputación Provincial de Sevilla, ITEA.

Andersson, L., Haley, C.S., Ellegren, H., Knott, S.A., Johansson, M., Andersson, K., Andersson-Eklund, L., Edfors-Lilja, I., Fredholm, M., Hansson, I., et al. 1994. Genetic mapping of quantitative trait loci for growth and fatness in pigs. Science. 263 (5154), 1771-4.

Andersson, L. 2001.Genetic dissection of phenotypic diversity in farm animals. Nature Reviews Genetics, 2, 130-138.

Archibald, A.L., Bolund, L., Churcher, C., Fredholm ,M., Groenen, M.A., Harlizius, B., Lee, K.T., Milan, D., Rogers, J., Rothschild, M.F., Uenishi, H., Wang, J., Schook, L.B. 2010. Swine

Genome Sequencing Consortium. Pig genome sequence analysis and publication strategy. BMC Genomics. 11: 438.

Ayuso, D., González, A., Hernández, F., Corral, JM., Izquierdo, M. 2013. Prediction of carcass composition, ham and foreleg weights, and lean meat yields of Iberian pigs using ultrasound measurements in live animals. Journal of Animal Science. 91, 1884-1892.

Ayuso, M., Fernández, A., Núñez, Y., Benítez, R., Isabel, B., Fernández, A.I., Rey, A.I., González-Bulnes, A., Medrano, J.F., Cánovas, Á., López-Bote, C.J., Óvilo, C. 2016. Developmental Stage, Muscle and Genetic Type Modify Muscle Transcriptome in Pigs: Effects on Gene Expression and Regulatory Factors Involved in Growth and Metabolism. Plos one. 11 (12): e0167858.

Barton-Gade, P.A. 1990. Pork quality in genetic improvement programmes – the Danish experience. Proc. Natl. Swine improv. Fed. Ann. Mtg. Des. Moines, Iowa, USA.

Béjar, F., Dobao, M.T., García-Casco, J.M., Rodrigáñez, J., Rodríguez, M.C., Silió, L., Toro, M.A., Campo, J.M., Rodríguez, R. y Sánchez, J. 1992. Investigaciones en la piara de cerdo Ibérico de "El Dehesón del Encinar". Servicio de Investigación y Experimentación Agraria. Área de Producción Animal. Núm. 6. Junta de Comunidades de Castilla-La Mancha.

Benítez, R., Trakooljul, N., Núñez, Y., Isabel, B., Murani, E., De Mercado, E., Gómez-Izquierdo, E., García-Casco, J., López-Bote, C., Wimmers, K., Óvilo, C. 2019. Breed, Diet, and Interaction Effects on Adipose Tissue Transcriptome in Iberian and Duroc Pigs Fed Different Energy Sources. Genes 10 (8), 589. https://doi.org/10.3390/genes10080589.

Bligh, E.G. and Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. 37, 911-917.

Boletín Oficial Estado, 2014. Real Decreto 4/2014, de 10 de enero, por el que se aprueba la norma de calidad para la carne, el jamón, la paleta y la caña de lomo ibérico. BOE nº 10, 1569-1585. https://www.boe.es/eli/es/rd/2014/01/10/4.

Boletín Oficial Estado, 2015. Ley 28/2015, de 30 de julio, para la defensa de la calidad alimentaria. BOE nº 182, 65884-65905. https://www.boe.es/eli/es/l/2015/07/30/28.

Boletín Oficial Estado, 2019. Real Decreto 45/2019, de 8 de febrero, por el que se establecen las normas zootécnicas aplicables a los animales reproductores de raza pura, porcinos reproductores híbridos y su material reproductivo, se actualiza el Programa nacional de conservación, mejora y fomento de las razas ganaderas y se modifican los Reales Decretos 558/2001, de 25 de mayo; 1316/1992, de 30 de octubre; 1438/1992, de 27 de noviembre; y 1625/2011, de 14 de noviembre.

Bonneau, M. and Lebret, B. 2010. Production systems and influence on eating quality of pork. Meat Science. 84, 293-300.

Bourne, M.C. 1978. Texture profile analysis. Food Technology, 32, 62–66, 72.

Cáceres-Nevado, J. M., Garrido-Varo, A., De Pedro-Sanz, E., Pérez-Marín, D. C. 2019. Fourier transform near-infrared spectroscopy coupled to a long fibre optic head for the quality control of IBERIAN pork loins: Intact versus minced. Meat Science, 153, 86-93. https://doi.org/10.1016/j.meatsci.2019.03.008.

Čandek-Potokar, M., Fontanesi, L., Lebret, B., Gil, J.M., Óvilo, C., Nieto, R., Fernandez, A., Pugliese, C., Oliver, M.A. and Bozzi, R. 2019. Chapter 1, Introductory chapter: Concept and Ambition of Project TREASURE. In book European local pig breeds-diversity and performance. A study of project TREASURE. DOI: 10.5772/intechopen.84246.

Cardoso, T.F., Cánovas, A., Canela-Xandri, O., González-Prendes, R., Amills, M., Quintanilla, R. 2017. RNA-seq based detection of differentially expressed genes in the skeletal muscle of Duroc pigs with distinct lipid profiles. Scientific reports. 7, 40005. doi: 10.1038/srep40005.

Carrapiso, A.I., Bonilla, F., García, C. 2003. Effect of crossbreeding and rearing system on sensory characteristics of Iberian ham. Meat science. 65, 623-629.

Chen, G., Wang, C., Shi, T. 2011. Overview of available methods for diverse RNASeq data analyses. Science China Life Sciences. 54, 1121-1128.

Clop, A., Óvilo, C., Pérez-Enciso, M., Cercós, A., Tomas, A., Fernández, A., Coll, A., Folch, J.M., Barragán, C., Díaz, I., Oliver, M.A., Varona, L., Silió, L., Sánchez, A., Noguera, J.L. 2003. Detection of QTL affecting fatty acid composition in the pig. Mammalian Genome. 14, 650-656.

Coenye, T. 2021. Do results obtained with RNA-sequencing require independent verification? Biofilm. Dec; 3: 100043. doi: 10.1016/j.bioflm.2021.100043.

Combes, S., Lepetit, J., Darche, B., Lebas, F. 2004. Effect of cooking temperature and cooking time on Warner-Bratzler tenderness measurement and collagen content in rabbit meat. Meat Science. 66 (1), 91–96. https://doi.org/10.1016/S0309 -1740(03)00019 -6.

Corominas, J., Ramayo-Caldas, Y., Puig-Oliveras, A., Pérez-Montarelo, D., Noguera, J.L., Folch, J.M., Ballester, M. 2013. Polymorphism in the ELOVL6 gene is associated with a major QTL effect on fatty acid composition in pigs. Plos One. 8 (1): e53687. doi: 10.1371/journal.pone.0053687.

Cozzolino, D., Murray, I., Scaife, J. R. Paterson, R. 2000. Study of dissected lamb muscles by visible and near infrared reflectance spectroscopy for composition assessment. Animal Science. 70, 417-423.

Crespo-Piazuelo, D., Criado-Mesas, L., Revilla, M., Castelló, A., Noguera, J. L., Fernández, A. I., Ballester, M., Folch, J. M. 2020. Identification of strong candidate genes for backfat and intramuscular fatty acid composition in three crosses based on the Iberian pig. Scientific reports. 10 (1), 1-17.

Damon, M., Wyszynska-Koko, J., Vincent, A., Hérault, F., Lebret, B. 2012. Comparison of muscle transcriptome between pigs with divergent meat quality phenotypes identifies genes related to muscle metabolism and structure. Plos one. 7, e33763.

Daza, A., López-Bote, C.J., Olivares, A., Menoyo, D., Ruiz, J. 2008. Influence of a severe reduction of the feeding level during the period immediately prior to free-range fattening on performance and fat quality in Iberian pigs. Journal of the science of food and agriculture. 88: 449-454.

de Hijas-Villalba, M.M., Varona, L., Ibáñez-Escriche, N., Rosas, J. P., Noguera, J. L., Casellas, J. 2021. Analysis of reproductive seasonality in Entrepelado and Retinto Iberian pig varieties under intensive management. Livestock Science, 245, 104441.

Diamond, J. 2002. Evolution, consequences and future of plant and animal domestication. Nature. 418, 700-707. https://doi.org/10.1038/nature01019.

Diéguez, E., 2000. La raza porcina Ibérica. Sólo Cerdo Ibérico, número 5.

Dobao, M. T., García Siles, J. L., de Pedro, E., Rodrigáñez, J., Sánchez, M and Silió, L. 1986. Diferencias en crecimiento y composición corporal entre cerdos ibéricos puros y cruzados con Duroc-Jersey. In Proceedings 9th Congress International Pig Veterinary Society, Barcelona, p. 382.

Dobao, M.T., Rodrigáñez, J., Silió, L., Toro, M.A. 1987. Implicaciones del cambio de estructura de población en la conservación del cerdo Ibérico. En: Campos, P. y Martín-Bellido, M. (Ed). Conservación y desarrollo de las dehesas portuguesas y españolas. Ministerio de agricultura. Madrid, p. 179-188.

Espárrago, F., Cabeza de Vaca, F., Molina, R. 1999. Censos y precios de porcino Ibérico. Sólo Cerdo Ibérico. Número 2, 113-122.

Esteve-Codina, A., Kofler, R., Himmelbauer, H., Ferretti, L., Vivancos, A.P., Groenen, M.A., Folch J.M., Rodríguez, M.C., Pérez-Enciso, M. 2011. Partial short-read sequencing of a highly inbred Iberian pig and genomics inference thereof. Heredity. 107 (3), 256-64. doi: 10.1038/hdy.2011.13.

Estévez, M., Morcuende, D., Cava López, R. 2003. Physicochemical characteristics of M. Longissimus dorsi from three lines of free-range reared Iberian pigs slaughtered at 90 kg live-weight and commercial pigs: A comparative study. Meat Science. 64 (4), 499–506. https://doi.org/10.1016/S0309 -1740(02)00228 -0

Fan, B., Du, Z.Q., Gorbach, D.M. and Rothschild, M.F. 2010. Development and application of high-density SNP arrays in genomic studies of domestic animals. Asian-Australasian Journal of Animal Sciences. 23, (7), 833 - 847.

Fang, M., Larson, G., Soares Ribeiro, H., Li, N., Andersson, L. 2009. Contrasting mode of evolution at a coat colour locus in wild and domestic pigs. Plos Genetics, 5 (1): e1000341. https://doi.org/10.1371/journal.pgen.1000341.

Fernández, A., De Pedro, E., Núñez, N., Silió, L., García Casco, J., Rodríguez, C. 2003. Genetic parameters for meat and fat quality and carcass composition traits in Iberian pigs. Meat Science. 64, 405-410.

Fernández, A. I., Pérez-Montarelo, D., Barragán, C., Ramayo-Caldas, Y., Ibáñez-Escriche, N., Castelló, A., Noguera, J.L., Silió, L., Folch, J.M., Rodríguez, M.C. 2012. Genome-wide linkage analysis of QTL for growth and body composition employing the PorcineSNP60 BeadChip. BMC genetics, 13 (1), 1-11.

Fernández-Rodríguez, A., Rodríguez, C., Varona, L., Balcells, I., Noguera, J. L., Óvilo, C., Fernández, A. I. 2010. Analysis of candidate genes underlying two epistatic quantitative trait loci on SSC12 affecting litter size in pig. Animal Genetics, 41 (1), 73-80.

Gangenahalli, G.U., Gupta, P., Saluja, D., Verma, Y.K., Kishore, V., Chandra, R., Sharma, R.K. Ravindranath, T. 2005. Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. Stem Cells and Development. 14 (2), 140-52.

García-Casco, J., Fernández, A., De Pedro, E., Rodríguez, C., Silió, L. 2008. Moderado antagonismo genético entre rendimiento en piezas nobles y contenido en grasa intramuscular en cerdos ibéricos. ITEA: Información Técnica Económica Agraria. vol. 104, nº 2, p.169-174.

García-Casco, J. M., Muñoz, M., Silió, L., Rodríguez-Valdovinos, C. 2014. Genotype by environment interaction for carcass traits and intramuscular fat content in heavy Iberian pigs fattened in two different free-range systems. Spanish Journal of Agricultural Research. 12: 388-395.

García-Casco, J.M. 2017. Programa de mejora para caracteres de ciclo completo y de calidad de carne en la finca La Contienda. Sólo Cerdo Ibérico, 37: 8 - 11.

García-Casco J.M., Fernández Barroso M.A., Caraballo C., Palma Granados P., López García A., Muñoz M., Ureta P., Burguillos A. Indiano E., Moreno P., García Valverde R., Martínez J. y Moreno B., Navarro S. 2020. Desarrollo del programa de evaluación genética para caracteres de crecimiento, ciclo completo y de calidad de carne en la finca La Contienda (2016-2019). Sólo Cerdo Ibérico, 43: 30-42.

Goddard, M.E., Hayes, B.J. 2009. Mapping genes for complex traits in domestic animals and their use in breeding programmes. Nature Reviews Genetics. 10 (6), 381-91.

Groenen, M., Archibald, A., Uenishi, H. et al. 2012. Analyses of pig genomes provide insight into porcine demography and evolution. Nature. 491, 393–398. https://doi.org/10.1038/nature11622.

Groenen, M.A. 2016. A decade of pig genome sequencing: a window on pig domestication and evolution. Genetics Selection and Evolution. 48, 23.

Groeneveld E., Kovak M., & Wang, T. 1999 Multivariate Prediction and Estimation v. 4.1. Department of Animal Sciences, University of Illinois. EEUU.

Groeneveld, E., Kovak, M., & Mielenz, N. 2010. Users' guide and reference manual, Version 6.0. Institute of farm animal genetics, Vol 6. VCE. Neustadt, Germany.

Hamill, R.M., McBryan, J., McGee, C., Mullen, A.M., Sweeney, T., Talbot, A., Cairns, M.T., Davey, G.C. 2012. Functional analysis of muscle gene expression profiles associated with tenderness and intramuscular fat content in pork. Meat Science. 92, 440–450.

Henderson, C. R. 1984. Applications of Linear Models in Animal Breeding. Guelph, Ontario: University of Guelph.

Hill, W. G. 2008. Estimation, effectiveness and opportunities of long-term genetic improvement in animals and maize. Lohmann Information. 43(1).

Hocquette, J.F., Gondret, F., Baza, E., Mdale, F., Jurie, C., Pethick, D.W. 2010. Intramuscular fat content in meat-producing animals: development, genetic and nutritional control, and identification of putative markers. Animal. 4, 303–319.

Honikel, K. 1998. Reference methods for the assessment of physical characteristics of meat. Meat Science. 49(4), 447–457.

Hopkins, D., Taylor, R. 2004. Post-mortem muscle proteolysis and meat tenderness. In muscle development of livestock animals: Physiology, genetics, and meat quality. Centre for agriculture and bioscience international. Wallingford, UK. pp. 363–388.

Hornsey, H. C. 1956. The colour of cooked cured pork. 1. Estimation of the nitric oxide haem pigments. Journal of the Science of Food and Agriculture. 7, 534–541.

Horodyska, J., Wimmers, K., Reyer, H., Trakooljul, N., Mullen, A.M., Lawlor, P.G., Hamill, R.M. 2018. RNA-Seq of muscle from pigs divergent in feed efficiency and product quality identifies differences in immune response, growth, and macronutrient and connective tissue metabolism. BMC Genomics. 19, 1–18.

Ibáñez-Escriche, N., Gonzalez-Recio, O. 2011. Review. Promises, pitfalls and challenges of genomic selection in breeding programs. Spanish Journal of Agricultural Research. 9 (2), 404-413.

Ibáñez-Escriche, N., Magallón, E., González, E., Tejeda, J.F., Noguera, J.L. 2016. Genetic parameters and crossbreeding effects of fat deposition and fatty acid profiles in Iberian pig lines. Journal of Animal Science. 94, 28-37.

Jones, G.F. 1998. Genetic aspects of domestication, common breeds and their origin. In The genetics of the pig (ed. MF Rothschild and A Ruvinsky), pp. 17–50. CABI Publishing, Wallingford, UK.

Kemp, C.M., Sensky, P.L., Bardsley, R.G., Buttery, P.J., Parr, T. 2010. Tenderness—An Enzymatic View. Meat Science. 84, 248–256.

Kennedy, B.W. 1990. Use of mixed model methodology in analysis of designed experiments. In: Gianola, D., Hammond, K. (eds). Advances in statistical methods for genetic improvement of livestock. Advanced series in agricultural sciences, 18. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-74487-7_5.

Knol, E.F., Nielsen, B., Knap, P.W. 2016. Genomic selection in commercial pig breeding. Animal Frontiers 6:15–22.

Koohmaraie, M., Geesink, G. H. 2006. Contribution of post-mortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. Meat Science, 74, 34–43.

Laguna-Sanz, E. 1998. El cerdo Ibérico. Madrid. Ediciones Mundi Prensa.

Larson, G., Dobney, K., Albarella, U., Fang, M, Matisoo-Smith, E., Robins, J., Lowden, S., Finlayson, H., Brand, T., Willerslev, E., Rowley-Conwy, P., Andersson, L., Cooper, A., 2005. Worldwide phylogeography of wild boar reveals multiple centers of pig domestication. Science. 307(5715), 1618-1621.

Lee, J.H., Song, K.D., Lee, H.K., Cho, K.H., Park, H.C., Park, K.D. 2015. Genetic parameters of reproductive and meat quality traits in Korean Berkshire pigs. Asian-Australasian Journal of Animal Science. 28, 1388–1393.

Legarra, A., Varona, L., de Maturana, E.L. 2008. TM Threshold Model; INRA-SAGA: Toulouse, France.

Li, S., Xu, X., Zhou, G. 2012. The roles of the actin-myosin interaction and proteolysis in tenderization during the aging of chicken muscle. Poultry Science. 91, 150–160.

Li, H., 2013. Systems genetics in “omics” era: current and future development. Theory Bioscience. 132 (1), 1-16.

Li, X.J., Zhou, J., Liu, L.Q., Qian, K., Wang C.L. 2016a. Identification of genes in longissimus dorsi muscle differentially expressed between Wannanhua and Yorkshire pigs using RNA-sequencing. Animal Genetics. 47 (3), 324-33.

Li, B., Liu, K., Weng, Q., Li, P., Wei, W., Li, Q., Chen, J., Huang, R., Wu, W., Liu, H. 2016b. RNA-seq analysis reveals new candidate genes for drip loss in a Pietrain x Duroc x Landrace x Yorkshire population. Animal Genetics. 47, 192-197.

Li, M., Chen, L., Tian, S., Lin, Y., Tang, Q., Zhou, X., Li, D., Yeung, CKL., Che, T., Jin, L., Fu, Y., Ma, J., Wang, X., Jiang, A., Lan, J., Pan, Q., Liu, Y., Luo, Z., Guo, Z., Liu, H., Zhu, L., Shuai, S., Tang, G., Zhao, J., Jiang, Y., Bai, L., Zhang, S., Mai, M., Li, C., Wang, D., Gu, Y., Wang, G., Lu, H., Li, Y., Zhu, H., Li, Z., Li, M., Gladyshev, V.N., Jiang, Z., Zhao, S., Wang, J., Li, R., Li, X. 2017. Comprehensive variation discovery and recovery of missing sequence in the pig genome using multiple de novo assemblies. Genome Research. 27 (5), 865-874.

Lillehammer, M., Meuwissen, T.H.E., Sonesson A.K. 2013. Genomic selection for two traits in a maternal pig breeding scheme. Journal of Animal Science. 91: 3079–3087.

Listrat, A., Lebret, B., Louveau, I., Astruc, T., Bonnet, M., Lefaucheur, L., Picard, B., Bugeon, J. 2016. How muscle structure and composition influence meat and flesh quality. Scientific World Journal. doi.org/10.1155/2016/3182746.

Liu, X., Xiong, X., Yang, J., Zhou, L., Yang, B., Ai, H., Ma, H., Xie, X., Huang, Y., Fang, S., Xiao, S., Ren, J., Ma, J., Huang, L. 2015. Genome-wide association analyses for meat quality traits in Chinese Erhualian pigs and a Western Duroc × (Landrace × Yorkshire) commercial population. Genetics Selection and Evolution. 47 (1), 44. doi: 10.1186/s12711-015-0120-x.

Lonergan, E. H., Lonergan, S.M. 2005. Mechanisms of water holding capacity of meat: the role of post-mortem biochemical and structural changes. Meat Science. 71 (1), 194–204.

López-Bote, C. J. 1998. Sustained utilization of the Iberian pig breed. Meat Science., 49, 17–27. doi.org/10.1016/S0309-1740(98)00072 -2.

López-Bote, C., Isabel, A., Isabel, R.B. 2001. Alimentación del cerdo ibérico en la dehesa. Porcino Ibérico: aspectos claves. Ed. Carlos Buxadé y Argimiro Daza. Mundiprensa. 217- 272.

Maltin, C., Balcerzak, D., Tilley, R., Delday, M. 2003. Determinants of meat quality: tenderness. Proceedings of the Nutrition Society. 62 (2): 337-47. doi: 10.1079/pns2003248

Mancini, R.A., Hunt, M.C. 2005. Current research in meat colour. Meat Science. 71, 100–21.

Martin, J.A, Wang, Z. 2011. Next-generation transcriptome assembly. Nature Reviews Genetics. 12 (10): 671-82.

Martínez-Montes, A. M., Fernández, A., Muñoz, M., Noguera, J. L., Folch, J. M., Fernández, A. I. 2018. Using genome wide association studies to identify common QTL regions in three different genetic backgrounds based on Iberian pig breed. Plos one. 13 (3), e0190184.

Melody, J.L., Lonergan, S.M., Rowe, L.J., Huiatt, T.W., Mayes, M. S., Huff-Lonergan, E. 2004. Early post-mortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles. Journal of Animal Science. 82, 1195–1205.

Meuwissen, T.H., Hayes, B.J., Goddard, M.E. 2001. Prediction of total genetic value using genome-wide dense marker maps. Genetics. 157 (4), 1819-29.

Meyer, K. 1991. DFRMEL-programs to estimate variance components by restricted maximum likelihood using a derivate-free approach. Version 2.0.

Milan, D., Jeon, J.T., Looft, C., Amarger, V., Robic, A., Thelander, M., Rogel-Gaillard, C., Paul, S., Iannuccelli, N., Rask, L., Ronne, H., Lundström, K., Reinsch, N., Gellin, J., Kalm, E., Roy, P.L., Chardon, P. Andersson, L. 2000. A mutation in PRKAG3 associated with excess glycogen content in pig skeletal muscle. Science. 288, 1248–1251.

Monroy, M., Prasher, S., Ngadi, M.O., Wang, N., Karimi, Y. 2010. Pork meat quality classification using Visible/Near-Infrared spectroscopic data. Biosystems Engineering. 107 (3), 271–276. doi:10.1016/j.biosystemseng.2010.09.006.

Monteiro-Balage, J., da Luz, S., Abdalla Gomide, C., de Nadai Bonin, M., Figueira, A.C. 2015. Predicting pork quality using Vis/NIR spectroscopy. Meat Science. 108, 37–43.

Morales, R., Guerrero, L., Serra, X., Gou, P. 2007. Instrumental evaluation of defective texture in dry-cured hams. Meat Science, 76 (3), 536–542.

Muñoz, G., Alves, E., Fernández, A., Ovilo, C., Barragán, C., Estellé, J., Quintanilla, R., Folch, J.M., Silió, L., Rodríguez, M.C., Fernández, A.I. 2007. QTL detection on porcine chromosome 12 for fatty-acid composition and association analyses of the fatty acid synthase, gastric inhibitory polypeptide and acetyl-coenzyme A carboxylase alpha genes. Animal Genetics. 38 (6), 639-46.

Muñoz, G., Óvilo, C., Silió, L., Tomás, A., Noguera, J.L., Rodríguez, M.C. 2009. Single- and joint-population analyses of two experimental pig crosses to confirm quantitative trait loci on Sus scrofa chromosome 6 and leptin receptor effects on fatness and growth traits. Journal of Animal Science. 87 (2):459-68.

Muñoz, M., Rodríguez, M. C., Alves, E., Folch, J. M., Ibáñez-Escriche, N., Silió, L., Fernández, A. I. 2013. Genome-wide analysis of porcine backfat and intramuscular fat fatty acid composition using high-density genotyping and expression data. BMC genomics, 14 (1), 1-15.

Muñoz, M., Bozzi, R., García, F., Núñez, Y., Geraci, C., Crovetti, A., García-Casco, J., Alves, E., Škrlep, M., Charneca, R., Martins, J.M., Quintanilla, R., Tibau, J., Kušec, G., Djurkin-Kušec, I., Mercat, M.J., Riquet, J., Estellé, J., Zimmer, C., Razmaite, V., Araujo, J.P., Radović, Č., Savić, R., Karolyi, D., Gallo, M., Čandek-Potokar, M., Fontanesi, L., Fernández, A.I., Óvilo,

C. 2018a. Diversity across major and candidate genes in European local pig breeds. Plos one, 13, e0207475. doi.org/10.1371/journal.pone.0207475.

Muñoz, M., García-Casco, J. M., Caraballo, C., Fernández-Barroso, M. A., Sánchez-Esquiliche, F., Gómez-Carballar, F., Rodríguez, C., Silió, L. 2018b. Identification of candidate genes and regulatory factors underlying intramuscular fat content through Longissimus dorsi transcriptome analyses in heavy Iberian pigs. Frontiers in Genetics, 682 (9), 1–16. https://doi.org/10.3389/fgene.2018.00608.

Muñoz, M., Sánchez-Esquiliche, F., Caraballo, C., Gómez, F., Pariente, J. M., Silió, L., Rodríguez, C., García-Casco, J.M. 2018c. Animal breeding scheme applied to the quality of pure Iberian Montanera pigs. Archivos de Zootecnia. 68, 9–11.

Muñoz, M., García-Casco, J. M., Alves, E., Benítez, R., Barragán, C., Caraballo, C., Fernández, A.I., García, F., Núñez, Y., Óvilo, C., Fernández, A., Rodríguez, C., Silió, L. 2020. Development of a 64 SNV panel for breed authentication in Iberian pigs and their derived meat products. Meat Science. 167, 108152. https://doi.org/10.1016/j.meats ci.2020.108152.

Murakami, M., Sato, H. Taketomi, Y. 2020. Updating Phospholipase A2. Biology Biomolecules. 10, 1457doi.org/10.3390/biom10101457.

Nagamine, Y., Haley, C.S., Sewalem, A., Visscher, P.M. 2003. Quantitative trait loci variation for growth and obesity between and within lines of pigs (Sus scrofa). Genetics. 164 (2): 629-35.

Noguera, J.L., Rodríguez, C., Varona, L., Tomàs, A., Muñoz, G., Ramírez, O., Barragán, C., Arqué, M., Bidanel, J.P., Amills, M., Óvilo, C., Sánchez, A. 2009. A bi-dimensional genome scan for prolificacy traits in pigs shows the existence of multiple epistatic QTL. BMC Genomics. 29 (10): 636. doi: 10.1186/1471-2164-10-636.

Núñez, Y., Radović, Č., Savić, R., García-Casco, J.M., Čandek-Potokar, M., Benítez, R., Radojković, D., Lukić, M., Gogić, M., Muñoz, M., Fontanesi, L., Óvilo, C. 2021. Muscle transcriptome analysis reveals molecular pathways related to oxidative phosphorylation, antioxidant defense, fatness and growth in Mangalitsa and Moravka pigs. Animals. 11 (3):844. doi: 10.3390/ani11030844.

Odriozola M. 1976. Investigación sobre los datos acumulados en dos piaras experimentales, Ministerio de Agricultura, IRYDA, 146 pp.

Olea, L., Miguel-Ayanz, A.S. 2006. The Spanish dehesa. A traditional Mediterranean silvopastoral system linking production and nature conservation. Grassland science in Europe. 11: 3-13.

Ollivier, L. 2009. European pig genetic diversity: A minireview. Animal. 3 (07), 915–924. https://doi.org/10.1017/s1751 731109004297.

Oshlack, A., Robinson, M.D., Young, M.D. 2010. From RNA-seq reads to differential expression results. Genome Biology. 11 (12):220.

Óvilo, C., Pérez-Enciso, M., Barragán, C., Clop, A., Rodríguez, C., Oliver, M.A., Toro, M.A., Noguera, J.L. 2000. A QTL for intramuscular fat and backfat thickness is located on porcine chromosome 6. Mammalian Genome. 11, 344-346.

Óvilo, C., Clop, A., Noguera, JL., Oliver, M.A., Barragán, C., Rodriguez, C., Silió, L., Toro, M.A., Coll, A., Folch, J.M., Sánchez, A., Babot, D., Varona, L., Pérez-Enciso, M. 2002. Quantitative trait locus mapping for meat quality traits in an Iberian x Landrace F2 pig population. Journal of Animal Science. 80 (11):2801-8. doi: 10.2527/2002.80112801x.

Óvilo, C., Fernández, A., Noguera, J., Barragán, C., Letón, R., Rodríguez, C., Toro, M. 2005. Fine mapping of porcine chromosome 6 QTL and LEPR effects on body composition in multiple generations of an Iberian by Landrace intercross. Genetical Research. 85 (1), 57-67. doi:10.1017/S0016672305007330.

Óvilo, C., Fernández, A.I., De Pedro, E., García-Casco, J., Rodríguez, C., Silió, L. 2006. Asociación de una mutación no sinónima del gen MC4R con el crecimiento y rendimiento de piezas nobles en cerdos ibéricos. Información Técnica Económica Agraria. 102: 79-85.

Óvilo, C., Fernández, A., Fernández, A. I., Folch, J. M., Varona, L., Benítez, R., Núñez, Y., Rodríguez, C., Silió, L. 2010. Hypothalamic expression of porcine leptin receptor (LEPR), neuropeptide Y (NPY), and cocaine- and amphetamine-regulated transcript (CART) genes is influenced by LEPR genotype. Mammalian Genome. 21 (11-12), 583–591. doi:10.1007/s00335-010-9307-1.

Óvilo, C., Benítez, R., Fernández, A., Núñez, Y., Ayuso, M., Fernández, A.I., Rodríguez, C., Isabel, B., Rey, A.I., López-Bote, C., Silió, L.. 2014. Longissimus dorsi transcriptome analysis of purebred and crossbred iberian pigs differing in muscle characteristics. BMC Genomics. 15, 413.

Pena, R.N., Quintanilla, R., Manunza, A., Gallardo, D., Casellas, J., Amills, M. 2014. Application of the microarray technology to the transcriptional analysis of muscle phenotypes in pigs. Animal Genetics. 45 (3): 311-21. doi: 10.1111/age.12146.

Pena, R.N., Noguera Jiménez, J.L., García-Santana, M.J., González, E., Tejeda, J. F., Ros Freixedes, R., Ibáñez-Escriche, N. 2019. Five genomic regions have a major impact on fat composition in Iberian pigs. Scientific Reports, 9 (1)2031. doi: 10.1038/s41598-019-38622-7.

Pérez-Enciso, M., Misztal, I. 2004. Qxpak: A versatile mixed model application for genetical genomics and QTL analyses. Bioinformatics, 20 (16), 2792–2798. https://doi.org/10.1093/bioinformatics/bth331.

Pérez-Marín, D.C., Garrido-Varo, A., Guerrero-Ginel, J.E., Gómez-Cabrera, A. 2004. Near-infrared reflectance spectroscopy (NIRS) for the mandatory labelling of compound feeding stuffs: chemical composition and open-declaration. Animal Feed Science and Technology. 116 (3-4), 333-349.

Pérez-Montarelo, D., Madsen, O., Alves, E., Rodríguez, M.C., Folch, J.M., Noguera, J.L., Groenen, M.A., Fernández, A.I. 2014. Identification of genes regulating growth and fatness traits in pig through hypothalamic transcriptome analysis. Physiological genomics. 46, 195-206.

Piórkowska, K., Zukowski, K., Ropka-Molik, K., Tyra, M. 2018. Detection of genetic variants between different polish landrace and puławska pigs by means of RNA-Seq analysis. Animal Genetics. 49, 21–225.

Ponsuksili, S., Murani, E., Trakooljul, N., Schwerin, M., Wimmers, K. 2014. Discovery of candidate genes for muscle traits based on GWAS supported by eQTL-analysis. International Journal of Biological Sciences. 10 (3): 327-37. doi: 10.7150/ijbs.8134.

Pugliese, C., Bozzi, R., Campodoni, G., Acciaioli, A., Franci, O., Gandini, G. 2005. Performance of Cinta Senese pigs reared outdoors and indoors. 1. Meat and subcutaneous fat characteristics. Meat Science. 69, 459–464.

Puig-Oliveras, A., Ramayo-Caldas, Y., Corominas, J., Estellé, J., Pérez-Montarelo, D., Hudson, N.J., Casellas, J., Folch, J.M., Ballester, M. 2014. Differences in muscle transcriptome among pigs phenotypically extreme for fatty acid composition. Plos One 9, 11.

Purslow, P.P. 2005. Intramuscular connective tissue and its role in meat quality. Meat Science. 70, 435–447.

Ramayo-Caldas, Y., Mercadé, A., Castelló, A., Yang, B., Rodríguez, C., Alves, E., Díaz, I., Ibáñez-Escriche, N., Noguera, J.L., Pérez-Enciso, M., Fernández, A.I., Folch, J. M. 2012. Genome-wide association study for intramuscular fatty acid composition in an Iberian× Landrace cross. Journal of animal science. 90 (9), 2883-2893.

Rey, A.I, Lopez-Bote, C.J. 2001. Effect of dietary copper and vitamin E supplementation, and extensive feeding with acorn and grass on longissimus muscle composition and susceptibility to oxidation in Iberian pigs. Journal of Animal Physiology and Animal Nutrition. 85: 281-292.

Rey, A.I., Daza, A., López-Carrasco, C., López-Bote, C. J. 2006. Quantitative study of the α- and γ-tocopherols accumulation in muscle and backfat from Iberian pigs kept free-range as affected by time of free-range feeding or weight gain. Animal Science, 82 (06), 901. https://doi.org/10.1017/asc20 06113.

Ripoll, G., Albertí, P., Panea, B., Olleta, J. L., Sañudo, C., 2008. Near-infrared reflectance spectroscopy for predicting chemical, instrumental and sensory quality of beef. Meat Science, 80 (3), 697–702.

Rivera-Ferre, M.G., Aguilera, J.F., Nieto, R. 2006. Differences in whole-body protein turnover between Iberian and Landrace pigs fed adequate or lysine-deficient diets. Journal of Animal Science, 84, 3346–55.

Rodríguez, C., Silió, L., Rodrigáñez, J. 2000. Balance de ocho años de funcionamiento del esquema de mejora genética de cerdos ibéricos. Sólo Cerdo Ibérico, número 5. 29-36.

Rodríguez, C., Tomás, A., Alves, E., Ramírez, O., Arqué, M., Muñoz, G., Barragán, C., Varona, L., Silió, L., Amills, M., Noguera, J.L. 2005. QTL mapping for teat number in an Iberian-by-Meishan pig intercross. Animal Genetics. 36 (6), 490-6. doi: 10.1111/j.1365-2052.2005.01358.x.

Rodríguez-Estévez, V., Sanchez-Rodriguez, M., Arce, C., García, A.R., Perea, J.M., Gustavo, A. 2012. Chapter 1: Consumption of acorns by finishing Iberian pigs and their function in the conservation of the dehesa agroecosystem. In book agroforestry for biodiversity and ecosystem services—Science and practice; Kaonga, M., Ed.; InTech: Rijeka, Croatia; pp. 1–22. DOI: 10.5772/2100.

Ruiz, J., López-Bote, C. 2002. Improvement of dry-cured ham quality by lipid modification through dietary means. In F. Toldra (Ed.), Research Advances in the Quality of Meat and Meat Products (pp. 255-271). Research Signpost.

SanCristobal, M., Chevalet, C., Haley, C.S., Joosten, R., Rattink, A.P., Harlizius, B., Groenen, M.A., Amigues, Y., Boscher, M.Y., Russell, G., Law, A., Davoli, R., Russo, V., Désautés, C., Alderson, L., Fimland, E., Bagga, M., Delgado, J.V., Vega-Pla, J.L., Martinez, A.M., Ramos, M., Glodek, P., Meyer, J.N., Gandini, G.C., Matassino, D., Plastow, G.S., Siggens, K.W., Laval, G., Archibald, A.L., Milan, D., Hammond, K., Cardellino, R. 2006. Genetic diversity within and between European pig breeds using microsatellite markers. Animal Genetics. 37 (3), 189–198. https://doi.org/10.1111/j.1365-2052.2005.01385.x.

Sellier, P. 1998. Genetics of Meat and Carcass Traits. Genetics of the Pig; Centre for Agriculture and Bioscience International: Wallingford, UK. pp. 463–510.

Serra, X., Gil, F., Pérez-Enciso, M., Oliver, M.A., Vázquez, J. M., Gispert, M., Noguera, J.L. 1998. A comparison of carcass, meat quality and histochemical characteristics of Iberian (Guadyerbas line) and Landrace pigs. Livestock Production Science, 56 (3), 215–223. https://doi.org/10.1016/S0301 -6226(98)00151 -1.

Serrano, M.P., Valencia, D.G., Nieto, M., Lázaro, R., Mateos, G.G. 2008. Influence of sex and terminal sire line on performance and carcass and meat quality of Iberian pigs reared under intensive production systems. Meat Science. 78 (4):420-8. doi: 10.1016/j.meatsci.2007.07.006.

Silió, L., Béjar, F., García-Casco, J.M., Rodríguez, C. Toro, M.A. 1992. Lo posible y lo deseable en la selección de cerdos Ibéricos. El cerdo Ibérico, la naturaleza, la dehesa. (Ed. Ministerio de Agricultura, Pesca y Alimentación), Ministerio de Agricultura, Pesca y Alimentación, Madrid.

Silió, L., Toro, M.A., Rodríguez, C., Rodríguez, J. 1997. Inferencias sobre cambios genéticos en una línea de cerdos Ibéricos seleccionada para crecimiento magro. Investigación agraria: Producción y sanidad animales. 12 (1, 2 y 3): 65-77.

Silió, L., Rodríguez, C., Rodrigáñez, J., Toro, M. 1999. Dos opciones para la mejora genética del tamaño de camada en cerdos Ibéricos. Sólo cerdo Ibérico. Número 3 (23-30).

Silió, L. 2000. Iberian pig breeding programme. In: Galal S, Boyazouglu J, Hammond K (eds) Developing Breeding Strategies for Lower Input Animal Production Environments. International Committee for Animal Recording: Roma, pp. 511–20.

Silió, L., Rodrigáñez, J. 2013. Una experiencia irrepetible: la piara de cerdos ibéricos de 'El Dehesón del Encinar' (1944-2012)". XV Jornadas sobre Producción Animal. Zaragoza. Tomo I, 3-16.

Silió, L., Rodríguez, M., Fernández, A., Barragán, C., Benítez, R., Óvilo, C., Fernández, A. 2013. Measuring inbreeding and inbreeding depression on pig growth from pedigree or SNP-derived metrics. Journal of Animal Breeding and Genetics. 130: 349-360. https://doi.org/10.1111/jbg.12031.

Solís, M., De Pedro, E., Garrido, A., García, J., Silió, L., Rodríguez, C., Rodrigáñez, J. 2001. Evaluación de la composición del lomo de cerdo ibérico mediante la tecnología NIRS. In Proceedings of the Asociación Interprofesional para el Desarrollo Agrario – ITEA (Vol. II, pp. 613–615). Zaragoza (Spain).

Szabo, E.F., Egnell, M., Galan, P., Druesne-Pecollo, N., Hercberg, S., Julia, C. 2019. Ability of the Nutri-Score front-of-pack nutrition label to discriminate the nutritional quality of foods in the German food market and consistency with nutritional recommendations. Archives of Public Health. 77:28. doi:10.1186/s13690-019-0357-x.

Tejeda, J.F., Gandemer, G., Antequera, T., Viau, M. and García, C. 2002. Lipid traits of muscles as related to genotype and fattening diet in Iberian pigs: total intramuscular lipids and triacylglycerols. Meat Science. 60, 357-363.

Tejerina, D., García-Torres, S., de Vaca, M.C., Vázquez, F., Cava, R. 2012a. Effect of production system on physical–chemical, antioxidant and fatty acids composition of Longissimus dorsi and Serratus ventralis muscles from Iberian pig. Food chemistry. 133, 293-299.

Tejerina, D., García-Torres, S., Cava, R. 2012b. Water-Holding capacity and instrumental texture properties of M. Longissimus dorsi and M. Serratus ventralis from Iberian Pigs as affected by the production system. Livestock Science, 148 (1–2), 46–51.

Tengvall, K., Bergvall, K., Olsson, M., Ardesjö-Lundgren, B., Farias, F.H.G., Kierczak, M., Hedhammar Å., Lindblad-Toh K., Andersson, G. 2020. Transcriptomes from German shepherd dogs reveal differences in immune activity between atopic dermatitis affected and control skin. Immunogenetics. 72 (5): 315-323.

Toro, M.A., Rodrigáñez, J., Rodríguez, C., Silió, L. 1995. Respuesta a la selección para crecimiento magro en una línea de cerdos Ibéricos. VI Jornadas sobre producción animal, ITEA. Tomo I, 236-238.

Toro, M. A., Rodrigáñez, J., Silió, L., Rodríguez, C. 2000. Genealogical analysis of a closed herd of black hairless Iberian pigs. Conservation Biology. 14, 1843–1851. https://doi.org/10.1111/j.1523-1739.2000.99322.x.

Torrescano, G., Sánchez-Escalante, A., Giménez, B., Roncalés, P., Beltrán, J.A. 2003. Shear values of raw samples of 14 bovine muscles and their relation to muscle collagen characteristics. Meat Science. 64, 85–91.

Ureta, P., Álvarez, F., Diéguez, E., Rodríguez, P., de la Rosa, R.S., Barandiarán, M., García-Casco J.M. 2010. Tendencias genéticas en las ganaderías participantes en el Índice de Lechón del Esquema de Valoración para la raza porcino Ibérica. Sólo Cerdo Ibérico. Número 23, 11-21.

Van Laere, A.S., Nguyen, M., Braunschweig, M., Nezer, C., Collette, C., Moreau, L., Archibald, A.L., Haley, C.S., Buys, N., Tally, M., Andersson, G., Georges, M., Andersson, L.A. 2003. Regulatory mutation in IGF2 causes a major QTL effect on muscle growth in the pig. Nature. 425 (6960), 832-6. doi: 10.1038/nature02064.

Varona, L., Noguera, J. L., Casellas, J., de Hijas, M. M., Rosas, J. P., Ibáñez-Escriche, N. 2020. A cross-specific multiplicative binomial recursive model for the analysis of perinatal mortality in a diallel cross among three varieties of Iberian pig. Scientific reports. 10 (1), 1-10.

Ventanas, S., Ventanas, J., Ruiz, J., Estevez, M. Iberian pigs for the development of high-quality cured products. Recent Res Develop Agricultural & Food Chem. 2005. 6 (27–53). Research Signpost, Kerala, India.

Ventanas, S., Estévez, M., Tejeda, J.F., Ruiz, J. 2006. Protein and lipid oxidation in Longissimus dorsi and dry cured loin from Iberian pigs as affected by crossbreeding and diet. Meat Science. 72, 647-655.

Ventanas, S., Ventanas, J., Ruiz, J. 2007. Sensory characteristics of Iberian dry cured loins: Influence of crossbreeding and rearing system. Meat science.75, 211-219.

Villaplana-Velasco, A., Noguera, J. L., Pena, R. N., Ballester, M., Muñoz, L., González, E., Tejeda, J.F., Ibáñez-Escriche, N. 2021. Comparative transcriptome profile between Iberian pig

varieties provides new insights into their distinct fat deposition and fatty acids content. Animals, 11(3), 627.

Wang, Q., Qi, R., Wang, J., Huang, W., Wu, Y., Huang, X., Yang, F. Huang J. 2017. Differential expression profile of miRNAs in porcine muscle and adipose tissue during development. Gene, 618, 49–56.

Warr, A., Affara, N., Aken, B., Beiki, H., Bickhart, D.M., Billis, K., Chow, W., Eory, L., Finlayson, H.A., Flicek, P., Girón, C.G., Griffin, D.K., Hall, R., Hannum, G., Hourlier, T., Howe, K., Hume, D.A., Izuogu, O., Kim, K., Koren, S., Liu, H., Manchanda, N., Martin, F.J., Nonneman, D.J., O'Connor, R.E., Phillippy, A.M., Rohrer, G.A., Rosen, B.D., Rund, L.A., Sargent, C.A., Schook, L.B., Schroeder, S.G., Schwartz, A.S., Skinner, B.M., Talbot, R., Tseng, E., Tuggle C.K., Watson, M., Smith, T.P.L., Archibald, A.L. 2020. An improved pig reference genome sequence to enable pig genetics and genomics research. Gigascience. 9 (6): giaa051. doi: 10.1093/gigascience/giaa051.

White, S. 2011. From Globalized pig breeds to capitalist pigs: a study in animals cultures and evolutionary history. Environmental History. 16, 94-120.

Wood, J.D. Consequences for Meat Quality of Reducing Carcass Fatness. In Reducing Fat in Meat Animals; Elsevier: Amsterdam, The Netherlands, 1990; pp. 344–397.

Wood, J.D., Enser, M., Fisher, A.V., Nute, G.R., Sheard, P.R., Richardson, R.I., Hughes, S.I. Whittington, F. M. 2008. Fat deposition, fatty acid composition and meat quality: A review. Meat Science, 78 (4), 343–358. doi:10.1016/j.meatsci.2007.07.019.

Xu, J., Wang, C., Jin, E., Gu, Y., Li, S., Li, Q. 2018. Identification of differentially expressed genes in longissimus dorsi muscle between Wei and Yorkshire pigs using RNA sequencing. Genes Genomics. 40 (4), 413-421.

Yu, Q.P., Feng, D.Y., Xiao, J., Wu, F., He, X.J., Xia, M.H., Dong, T., Liu, Y.H., Tan, H.Z., Zou, S.G., Zheng, T., Ou, X.H. and Zuo, J.J. 2017. Studies on meat colour, myoglobin content, enzyme activities, and genes associated with oxidative potential of pigs slaughtered at different growth stages. Asian-Australasian Journal of Animal Science. 30, 12:1739-1750. https://doi.org/10.5713/ajas.17.0005.

Zamora-Rojas, E., Garrido-Varo, A., De Pedro-Sanz, E., Guerrero-Ginel, J. E., Pérez-Marín, D. 2011. Monitoring NIRS calibrations for use in routine meat analysis as part of Iberian pig-breeding programs. Food Chemistry. 129, 1889–1897.

Zamora-Rojas, E., Pérez-Marín, D., De Pedro-Sanz, E., Guerrero-Ginel, J. E., Garrido- Varo, A., 2012. In-situ Iberian pig carcass classification using a micro-electro-mechanical system (MEMS)-based near infrared (NIR) spectrometer. Meat Science. 90, 636–642.

Zappaterra, M., Gioiosa, S., Chillemi, G., Zambonelli, P., Davoli, R. 2020. Muscle transcriptome analysis identifies genes involved in ciliogenesis and the molecular cascade associated with intramuscular fat content in Large White heavy pigs. Plos one. 15, e0233372.

Zhang, C., Wang, Z., Bruce, H., Kemp, R.A., Charagu, P., Miar, Y., Yang, T., Plastow, G. 2015. Genome-wide association studies (GWAS) identify a QTL close to PRKAG3 affecting meat pH and colour in crossbred commercial pigs. BMC Genetetics. 7 (16), 33. doi: 10.1186/s12863-015-0192-1.

Zhu, J., Shi, X.E., Lu, H., Xia, B., Li, Y., Li, X., Zhang, Q., Yang, G. 2016. RNA-Seq transcriptome analysis of extensor digitorum longus and soleus muscles in Large White pigs. Molecular Genetics and Genomics. 291, 687–701.

Zimmerman, A.W., Veerkamp, J.H. 2002. New Insights into the structure and function of fatty acid-binding proteins. Cellular and Molecular Life Sciences. 59, 1096–1116.

ANEXO

MATERIAL SUMPLEMENTARIO

CAPÍTULO 1. Genetic parameter estimation and gene association analyses for meat quality traits in open-air free-range Iberian pigs.

Supplementary Table 1. Primer name, sequences, size, melting Temperature (TM) and amplicon size of genes selected for expression analyses.

Name	Sequence 5'3'	Primer Size	T_M	Amplicon Size
CAPN1F	GGCTTTGCCGTCTATGAGGT	20	60.11	241
CAPN1R	CTTGGGTCCCGGCTTTCTTC	20	60.96	
PRKAG3F	ATCAAGAACCGGATCCACCG	20	59.82	156
PRKAG3R	GCCCAAATCTTGGATGGTGC	20	59.82	

Supplementary Table 2. Genetic correlation estimates of shear force for cook meat (SFF), centrifuge force losses (CFL), thawing loss (TL), cooking loss (CL) and Minolta instrumental colour traits (L*, a*, b*).

	Tenderness	WHC		
	SFF	**TL**	**CL**	**CFL**
L*	0.39 (0.18)	0.54 (0.08)	0.72 (0.07)	0.76 (0.25)
a*	0.34 (0.25)	0.74 (0.07)	0.26 (0.11)	0.88 (0.13)
b*	0.42 (0.22)	0.94 (0.02)	0.70 (0.11)	0.95 (0.10)

WHC: water holding capacity

Supplementary Table 3. Allelic and genotypic frequencies for significant polymorphisms on candidate genes for meat quality.

SNP	A_1	A_2	A_1A_1	A_1A_2	A_2A_2
CAPN1_rs196954097G>C	0.08 (G)	0.92 (C)	0.00 (GG)	0.15 (GC)	0.85 (CC)
CAPN1_rs81358636G>A	0.17 (G)	0.83 (A)	0.02 (GG)	0.30 (GA)	0.68 (AA)
CAPN1_rs81358667G>A	0.37 (G)	0.63 (A)	0.13 (GG)	0.48 (GA)	0.39 (AA)
CAPN3_rs323177300C>T	0.94 (C)	0.06 (T)	0.89 (CC)	0.11 (CT)	0.00 (TT)
CAPNS1_rs329615471A>C	0.90 (A)	0.10 (C)	0.80 (AA)	0.20 (AC)	0.00 (CC)
CAPNS1_rs346167312C>G	0.90 (C)	0.10 (G)	0.81 (CC)	0.19 (CG)	0.00 (GG)
CAPNS1_rs789976262G>A	0.75 (T)	0.25 (A)	0.55 (TT)	0.38 (TA)	0.06 (AA)
CAPNS2_rs323301722C>T	0.79 (C)	0.21 (T)	0.62 (CC)	0.34 (CT)	0.04 (TT)
CASP3_rs319658214G>T	0.33 (G)	0.67 (T)	0.10 (GG)	0.47 (GT)	0.43 (TT)
CASP3_rs321490445C>A	0.90 (C)	0.10 (A)	0.81(CC)	0.18 (CA)	0.01 (AA)
CASP9_rs324307617C>T	0.22 (C)	0.78 (T)	0.05 (CC)	0.34 (CT)	0.61 (TT)
CASP9_rs346077419G>A	0.22 (G)	0.78 (A)	0.05 (GG)	0.34 (GA)	0.61 (AA)
CASP9_rs346188168G>A	0.92 (G)	0.08 (A)	0.84 (GG)	0.16 (GA)	0.01 (AA)
CTSB_rs326566953A>G	0.67 (A)	0.33 (G)	0.43 (AA)	0.48 (AG)	0.09 (GG)
CTSB_rs342116151G>A	0.46 (G)	0.54 (A)	0.20 (GG)	0.51 (GA)	0.28 (AA)
CTSB_rs344637144G>C	0.54 (G)	0.46 (C)	0.28 (GG)	0.51 (GC)	0.20 (CC)
CTSB_rs81210988G>A	0.54 (G)	0.46 (A)	0.28 (GG)	0.51 (GA)	0.20 (AA)
CTSL_rs321623592A>T	0.36 (A)	0.64 (T)	0.13 (AA)	0.46 (AT)	0.41 (TT)
CTSL_rs332171512A>G	0.53 (A)	0.47 (G)	0.27 (AA)	0.52 (AG)	0.21 (GG)
CTSL_rs340013902T>C	0.23 (T)	0.77 (C)	0.04 (TT)	0.39 (TC)	0.57 (CC)
MYH1_rs339679793C>T	1 (C)	0 (TT)	1 (CC)	0 (CT)	0 (TT)
MYH1_rs341385020C>T	1 (C)	0 (TT)	1 (CC)	0 (CT)	0 (TT)
MYH1_rs695620698A>G	1 (A)	0 (GG)	1 (AA)	0 (AG)	0 (GG)
MYH3_rs330483504C>A	0.57 (C)	0.43 (A)	0.33 (CC)	0.49 (CA)	0.18 (AA)
MYH3_rs333004392G>T	0 (G)	1 (TT)	0 (GG)	0 (CT)	1 (TT)
MYH3_rs341130038A>C	0.57 (A)	0.43 (C)	0.33 (AA)	0.49 (AC)	0.19 (CC)
MYH3_rs81211437A>G	0.57 (A)	0.43 (G)	0.32 (AA)	0.49 (AG)	0.19 (GG)
MYH3_rs81437544T>C	0.43 (T)	0.57 (C)	0.18 (TT)	0.49 (TC)	0.33 (CC)
MYOD_rs345162462T>C	0.42 (T)	0.58 (C)	0.17 (TT)	0.50 (TC)	0.33 (CC)
PRKAG3_rs319678464G>C	0.68 (G)	0.32 (C)	0.46 (GG)	0.45 (GC)	0.09 (CC)
PRKAG3_rs330427832C>T	0.68 (C)	0.32 (T)	0.46 (CC)	0.45 (CT)	0.09 (TT)
PRKAG3_rs343733804G>A	Genotyping failed				

Supplementary Table 4. SNPs affecting shear force in fresh loin with *p-value* lower than 0.05.

SNP	a (SE)	PM	LR	p-value	q-value
Shear Force (kg/cm²)					
CAPN1_rs81358636G>A	-0.23(0.06)	-5.31	11.24	$7.99x10^{-4}$	0.005
CAPN1_rs81358667G>A	-0.24 (0.05)	-5.54	20.89	$4.86x10^{-6}$	$1.31x10^{-4}$
CAPN3_rs323177300C>T	0.28 (0.11)	6.47	6.65	0.010	0.050
CAPNS2_rs323301722C>T	0.22 (0.06)	5.08	11.39	$7.39x10^{-4}$	0.005
CASP3_rs319658214G>T	-0.25 (0.06)	-5.77	17.88	$2.35x10^{-5}$	$3.17x10^{-4}$
CASP9_rs346188168G>A	0.21 (0.09)	4.85	5.43	0.020	0.080

a: additive effect; SE: standard error; PM: additive effect expressed as a percentage of the mean of the trait; LR: likelihood ratio test values.

Supplementary Table 5. SNPs affecting the percentage of thawing, cooking and centrifuge force losses with p-value lower than 0.05.

SNP	a (SE)	PM	LR	p-value	q-value
Thawing Loss (%)					
CAPN1_rs81358636G>A	-0.41 (0.19)	-4.34	5.36	0.021	0.069
CAPN3_rs323177300G>A	0.61 (0.29)	6.46	4.81	0.028	0.083
CAPNS1_rs329615471A>C	0.57 (0.24)	6.03	6.38	0.012	0.045
CAPNS1_rs346167312C>G	0.47 (0.24)	4.97	4.49	0.034	0.083
CAPNS2_rs323301722C>T	0.49 (0.17)	5.19	8.66	0.003	0.018
CASP3_rs319658214G>T	-0.51 (0.16)	-5.40	11.84	$5.81x10^{-4}$	0.004
CASP9_rs324307617C>T	-0.32 (0.17)	-3.39	4.10	0.043	0.084
CASP9_rs346077419G>A	-0.32 (0.17)	-3.39	4.06	0.044	0.084
CASP9_rs346188168G>A	0.83 (0.24)	8.78	11.99	$5.35x10^{-4}$	0.004
CTSL_rs321623592A>T	0.38 (0.15)	4.02	6.82	0.009	0.041
CTSL_rs332171512A>G	0.29 (0.15)	3.07	4.30	0.038	0.084
MYOD_rs345162462T>C	-0.33 (0.15)	-3.49	4.57	0.033	0.083
PRKAG3_rs319678464G>C	-0.88 (0.16)	-9.31	28.61	$8.86x10^{-8}$	$1.29x10^{-6}$
PRKAG3_rs330427832C>T	-0.88 (0.16)	-9.31	28.46	$9.55x10^{-8}$	$1.29x10^{-6}$
Cooking Loss (%)					
CAPN1_rs81358667G>A	-0.32 (0.14)	-1.33	5.07	0.020	0.070
CAPNS2_rs323301722C>T	0.56 (0.17)	2.32	10.04	0.002	0.006
CASP3_rs319658214G>T	-0.49 (0.16)	-2.03	10.00	0.002	0.006
MYH3_rs330483504C>A	0.50 (0.15)	2.07	11.74	$6.11x10^{-4}$	0.004
MYH3_rs341130038A>C	0.49 (0.15)	2.03	11.40	$7.34x10^{-4}$	0.004
MYH3_rs81211437A>G	0.47 (0.15)	1.95	9.22	0.002	0.008
MYH3_rs81437544T>C	-0.50 (0.15)	2.07	11.67	$6.35x10^{-4}$	0.004
PRKAG3_rs319678464G>C	-1.02 (0.16)	-4.23	41.91	$9.55x10^{-11}$	$1.29x10^{-9}$
PRKAG3_rs330427832C>T	-1.04 (0.16)	-4.31	42.09	$8.72x10^{-11}$	$1.29x10^{-9}$
Centrifuge force losses					
CAPN1_rs81358667G>A	-0.45 (0.17)	-1.38	6.93	0.008	0.038
CAPNS1_rs329615471A>C	-0.76 (0.34)	-2.33	4.80	0.028	0.096
CAPNS1_rs789976262G>A	0.61 (0.23)	1.87	7.16	0.007	0.038
CTSL_rs321623592A>T	0.46 (0.17)	1.41	7.04	0.008	0.038
CTSL_rs332171512A>G	0.59 (0.17)	1.81	11.90	$5.62x10^{-4}$	0.005
CTSL_rs340013902T>C	0.42 (0.21)	1.29	3.93	0.048	0.143
MYOD_rs345162462T>C	-0.46 (0.18)	-1.41	6.31	0.012	0.046
PRKAG3_rs319678464G>C	-0.83 (0.19)	-2.55	19.43	$1.04x10^{-5}$	$2.81x10^{-4}$
PRKAG3_rs330427832C>T	-0.78 (0.19)	-2.39	17.99	$2.22x10^{-5}$	$3.00x10^{-4}$

a: additive effect; SE: standard error; PM: additive effect expressed as a percentage of the mean of the trait; LR: likelihood ratio test values.

Supplementary Table 6. SNPs affecting Minolta instrumental parameters and myoglobin content with *p-value* lower than 0.05.

SNP	a (SE)	PM	LR	p-value	q-value
L*					
CAPN1_rs196954097G>C	0.52 (0.25)	1.30	4.29	0.038	0.147
CTSB_rs326566953A>G	0.35 (0.15)	0.87	5.95	0.015	0.071
CTSL_rs321623592A>T	0.43 (0.14)	1.07	9.39	0.002	0.034
CTSL_rs332171512A>G	0.38 (0.14)	0.95	7.47	0.006	0.056
MYOD_rs345162462T>C	-0.44 (0.15)	-1.10	9.14	0.003	0.034
PRKAG3_rs319678464G>C	-0.38 (0.15)	-0.95	6.26	0.012	0.071
PRKAG3_rs330427832C>T	-0.37 (0.15)	-0.92	5.84	0.015	0.071
a*					
CASP9_rs346188168G>A	0.29 (0.11)	2.85	6.91	0.009	0.077
PRKAG3_rs319678464G>C	-0.34(0.07)	-3.34	23.27	$1.4x10^{-6}$	$1.89x10^{-5}$
PRKAG3_rs330427832C>T	-0.34(0.07)	-3.34	23.66	$1.15x10^{-6}$	$1.89x10^{-5}$
b*					
CAPNS1_rs789976262G>A	0.23 (0.06)	3.58	14.50	$1.4x10^{-4}$	0.001
CASP3_rs321490445C>A	0.19 (0.08)	2.95	6.96	0.008	0.045
CASP9_rs346188168G>A	0.22 (0.08)	3.42	6.99	0.008	0.045
CTSL_rs332171512A>G	0.11 (0.05)	1.71	5.31	0.021	0.082
MYOD_rs345162462T>C	-0.12(0.05)	-1.87	6.44	0.011	0.050
PRKAG3_rs319678464G>C	-0.27 (0.05)	-4.20	26.03	$3.37x10^{-7}$	$7.64x10^{-6}$
PRKAG3_rs330427832C>T	-0.27 (0.05)	-4.20	25.03	$5.66x10^{-7}$	$7.64x10^{-6}$
Myoglobin content					
CASP9_rs324307617C>T	0.05 (0.02)	2.82	8.96	0.003	0.047
CASP9_rs346077419G>A	0.05 (0.02)	2.82	8.54	0.003	0.047
MYH3_rs330483504C>A	0.03 (0.01)	1.69	3.99	0.046	0.177
MYH3_rs341130038A>C	0.03 (0.01)	1.69	4.48	0.034	0.177
MYH3_rs81437544T>C	-0.03 (0.01)	-1.69	4.09	0.043	0.177
PRKAG3_rs319678464G>C	-0.04 (0.02)	-2.26	6.57	0.010	0.070
PRKAG3_rs330427832C>T	-0.04 (0.02)	-2.26	7.05	0.008	0.070

a: additive effect; SE: standard error; PM: additive effect expressed as a percentage of the mean of the trait; LR: likelihood ratio test values.

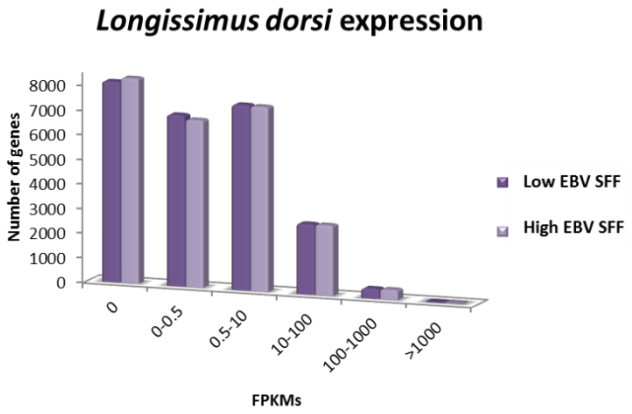
Supplementary Table 7. SNPs affecting dry-cured samples shear force and maximum compression force in dry-cured loins with *p-value* lower than 0.05.

SNP	a (SE)	PM	LR	p-value	q-value
Dry-cured Shear force (kg/cm^2)					
CAPNS1_rs329615471A>C	0.49 (0.21)	6.56	5.73	0.02	0.36
PRKAG3_rs330427832C>T	0.23 (0.11)	3.08	4.93	0.03	0.36
Maximum compression force (kg/cm^2)					
CAPN1_rs196954097G>C	-0.33 (0.17)	-6.11	4.25	0.04	0.53
CAPNS1_rs329615471A>C	0.45 (0.17)	8.33	6.47	0.01	0.29

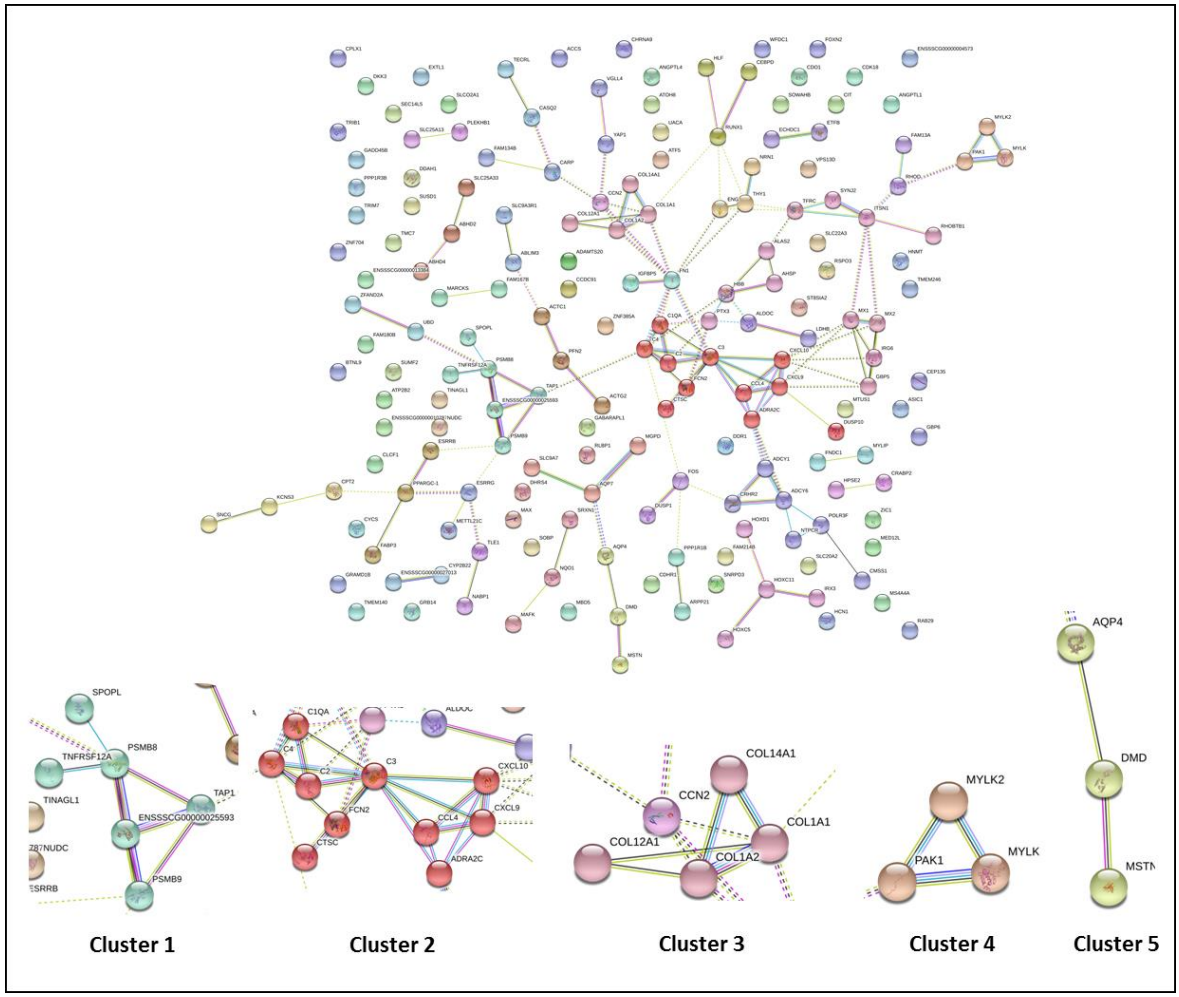
a: additive effect; SE: standard error; PM: additive effect expressed as a percentage of the mean of the trait; LR: likelihood ratio test values.

CAPÍTULO 3: Differences in loin tenderness of Iberian pigs explained through dissimilarities in their transcriptome expression profile.

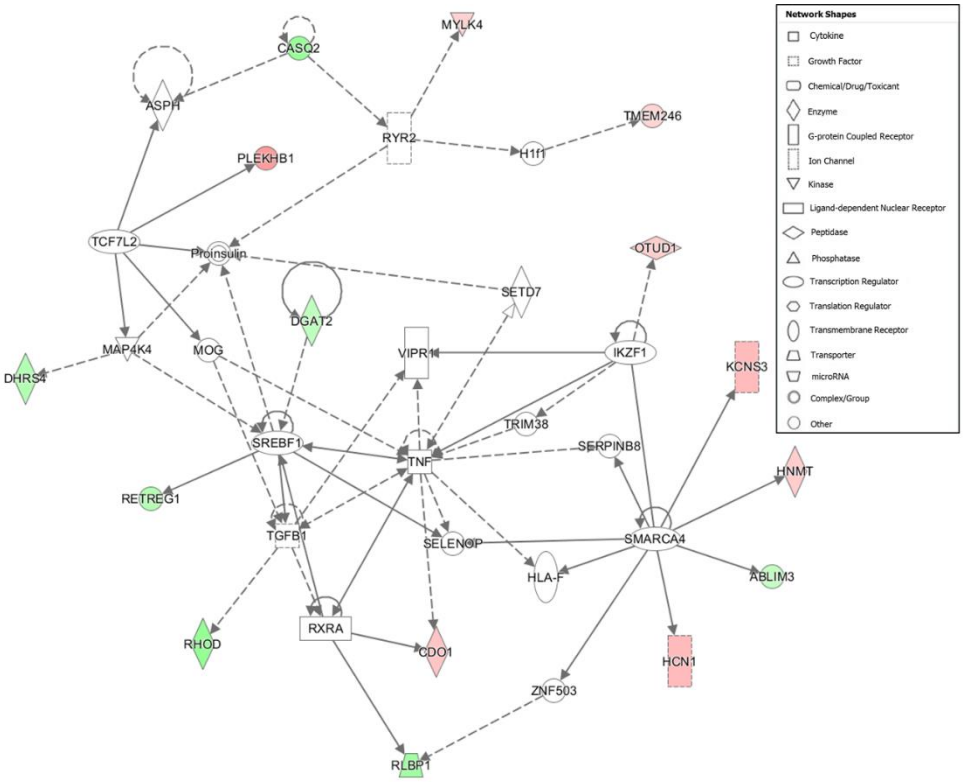
Supplementary Figure 1: Gene expression distribution of the 25,878 genes annotated in the pig genome (Sscrofa11.1) in fragments per kilobase of transcript per million mapped fragments (FPKMs) normalized values corresponding to the animals with the highest EBV for shear force and tougher meat (High EBV SFF) and the lowest EBV for shear force and tenderer meat (Low EBV SFF).



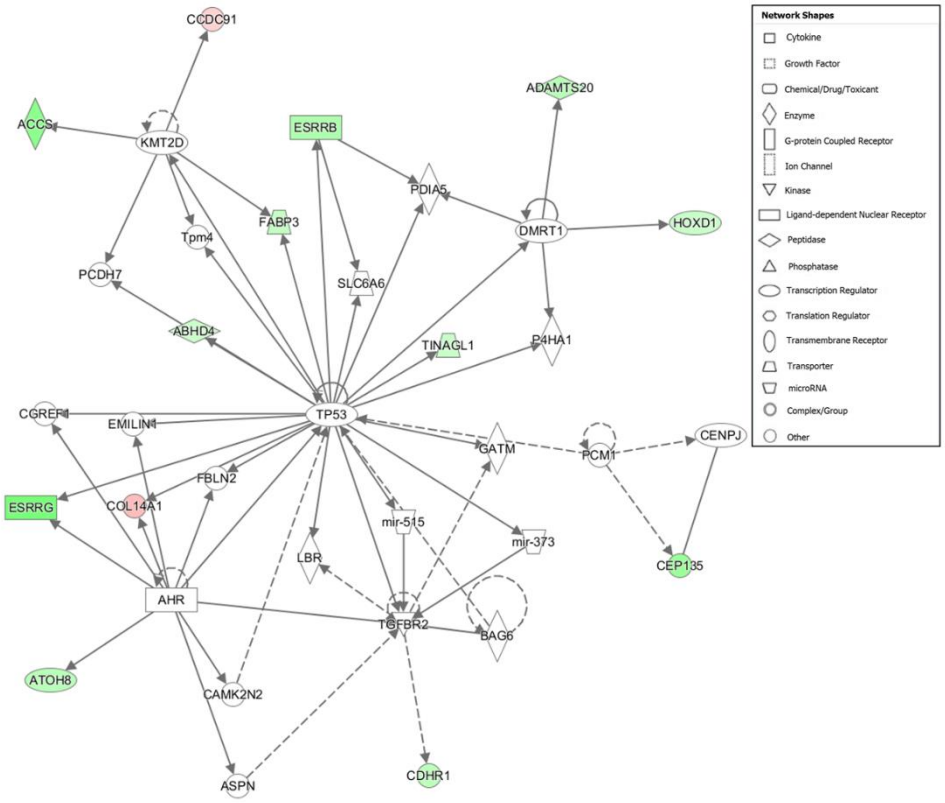
Supplementary Figure 2: Network of protein–protein interactions predicted with the STRING database. Same color nodes sharing multiple edges are grouped in the same cluster.



Supplementary Figure 3: Gene network #8: Cardiovascular System Development and Function, Cell Cycle, Gene Expression. Genes upregulated and down-regulated in the Tender group are represented in green and red colors, respectively.



Supplementary Figure 4: Gene network #9: Cell Cycle, Cellular Assembly and Organization, Cellular Movement. Genes upregulated and down-regulated in the Tender group are represented in green and red colors, respectively.



CAPÍTULO 4. Understanding the role of myoglobin content in Iberian pigs fattened in an extensive system through analysis of the transcriptome profile.

Supplementary Table 1: Primer name, sequences, melting temperature (TM) and amplicon size of genes selected for qPCR validation.

Name	Sequence 5'3'	T_M	Amplicon Size
CD209F	TGAAGGTTCCTGGCAATGGG	60.25	169
CD209R	AGACGAGGGCTTCTCACAGA	60.25	
FESF	CACCAGGAGATGGCGAACAT	60.11	212
FESR	TCAAGCAGCGACTCGTCAAA	60.25	
PLA2G10F	GCAGCTTCCCAAAGGTCACA	60.82	74
PLA2G10R	GTGCCAACGCAATTCACAGT	50.00	
ZSCAN31F	GGGCCTCAAAAACTTGGAGA	58.00	193
ZSCAN31R	TGGGGTGTCCCCTCTGATTT	60.48	
DGAT2F	CGTGCTGCGGGAGTACCTGATGTC	71.50	123
DGAT2R	TCGGCTGCACCACCCACCAC	72.50	
ATP6F	CTTTTATTGCCCCCACGAT	61.10	210
ATP6R	ATGTTTGTTGAGCCGATGAATA	61.60	

Supplementary Table 2: Total number of reads, filtered reads and percentage of mapped reads per sample.

Type	Total Reads, n	Filtered Reads, n	Uniquely mapped, %	Mapped in more than one position, %	Unmapped, %	Mapped Results, %
High Mb_1	95,758,844	95,707,408	87.02	4.78	8.20	91.80
High Mb_2	93,414,834	93,379,136	88.46	4.01	7.53	92.47
High Mb_3	92,547,312	92,509,896	87.34	5.29	7.38	92.62
High Mb_4	140,757,990	140,666,628	87.92	5.71	6.37	93.63
High Mb_5	95,290,300	95,244,082	87.23	4.59	8.19	91.81
High Mb_6	215,238,920	215,096,684	88.06	5.87	6.06	93.94
Low Mb_1	174,361,280	174,265,972	87.81	6.18	6.01	93.99
Low Mb_2	92,544,226	92,508,488	88.01	4.92	7.07	92.93
Low Mb_3	92,953,358	92,905,028	87.35	4.90	7.75	92.25
Low Mb_4	93,542,106	93,496,812	87.64	5.16	7.20	92.80
Low Mb_5	16,3842,102	163,741,822	88.13	5.67	6.21	93.79
Low Mb_6	148,214,322	148,139,252	88.01	6.06	5.93	94.07

High MB_1-6: Individuals with high breeding value for myoglobin content. Low MB_1-6: Individuals with low breeding value for myoglobin content.

Supplementary Table 3. Fold change, expression average of the High and Low myoglobin groups, p-Value and q-Value corresponding to the set of differentially expressed genes (DEGs) and transcripts.

Gene	Log_2 FC	High[a]	Low[a]	*p-Value*	*q-Value*
ECH1	1.257	27.562	66.176	1.33×10^{-04}	0.093
ZSCAN31	0.844	55.912	99.990	6.23×10^{-04}	0.085
RAB11FIP5	0.620	458.569	705.502	7.97×10^{-05}	0.066
CCDC191	0.607	112.028	170.700	2.21×10^{-04}	0.072
TRIM47	-0.657	418.485	266.034	3.38×10^{-04}	0.085
P2RX7	-0.663	142.904	90.183	6.39×10^{-04}	0.086
GPIHBP1	-0.663	454.975	287.171	3.97×10^{-04}	0.089
PLD2	-0.676	246.866	153.997	9.13×10^{-06}	0.007
SH3BGRL3	-0.682	651.613	405.638	1.61×10^{-05}	0.020
EPB41L1	-0.726	346.906	208.891	5.37×10^{-04}	0.093
DEPDC1B	-0.730	257.177	154.687	5.40×10^{-04}	0.083
PRCP	-0.730	423.353	255.123	4.30×10^{-04}	0.089
COL12A1	-0.734	286.057	172.327	8.69×10^{-05}	0.055
NOVA1	-0.747	236.285	140.854	1.47×10^{-04}	0.047
FBLN1	-0.807	894.723	511.670	1.29×10^{-04}	0.069
IFI44	-0.814	147.531	83.185	5.39×10^{-04}	0.085
FES	-0.817	251.164	142.776	2.13×10^{-04}	0.065
MX2	-0.824	186.233	104.884	6.74×10^{-04}	0.089
SLA-2	-0.852	14004.763	7759.332	1.00×10^{-05}	0.025
ENSSSCG00000027790	-0.862	113.477	62.768	4.34×10^{-05}	0.022
EPB41L3	-0.884	107.260	58.216	1.49×10^{-04}	0.043
HES6	-0.890	89.813	47.896	9.64×10^{-04}	0.089
MGP	-0.908	1990.586	1061.537	1.50×10^{-04}	0.098
IFI44L	-0.916	347.766	184.011	1.22×10^{-06}	0.004
ENSSSCG00000034739	-0.925	68.839	36.248	5.63×10^{-04}	0.077
EFEMP1	-0.930	570.677	299.524	4.36×10^{-05}	0.029
MFAP2	-0.961	150.201	77.709	4.72×10^{-04}	0.089
PLA2G10	-0.971	91.443	46.570	3.15×10^{-04}	0.069
LGALS9	-0.982	175.177	88.313	3.82×10^{-05}	0.027
LGALS3	-1.031	371.154	181.950	3.22×10^{-05}	0.025
IFI6	-1.079	741.529	350.471	3.81×10^{-04}	0.089
putative MORF4 family-associated protein 1-like protein UPP	-1.096	144.164	67.304	3.50×10^{-04}	0.072
CRIP1	-1.127	270.734	124.242	3.74×10^{-05}	0.027
BICD1	-1.254	38.452	16.344	1.82×10^{-04}	0.098
SLA-5	-1.275	443.374	182.534	2.98×10^{-05}	0.025
FNDC1	-1.280	134.918	55.835	2.97×10^{-04}	0.083
HBA1	-1.285	181.414	73.893	4.97×10^{-07}	0.002
SNORA53	-1.294	35.670	14.461	2.56×10^{-04}	0.086
ACKR2	-1.333	95.303	38.195	2.11×10^{-04}	0.066
SLA-7	-1.371	328.055	126.352	3.47×10^{-04}	0.085
FMO5	-1.379	39.468	15.044	4.17×10^{-04}	0.086
SLC16A7	-1.383	95.706	36.696	5.36×10^{-04}	0.083
RPRM	-1.410	71.568	27.016	1.11×10^{-03}	0.094

ACKR4	-1.437	137.955	50.912	9.81×10^{-05}	0.051
ENSSSCT00000059693	-1.478	152.229	54.143	2.52×10^{-05}	0.042
TRIM6	-1.634	73.803	23.459	7.49×10^{-06}	0.007
ENSSSCG00000033858	-1.707	145.668	44.641	4.58×10^{-04}	0.089
SCIN	-1.724	37.364	11.254	2.66×10^{-04}	0.077
BASP1	-1.733	86.036	25.952	1.75×10^{-04}	0.047
CD209	-1.857	342.653	94.712	3.44×10^{-06}	0.006
VSTM1	-1.958	25.533	6.619	1.97×10^{-04}	0.069
ENSSSCG00000034089	-2.008	29.037	7.385	5.65×10^{-04}	0.094
ENSSSCG00000038322	-3.713	27.051	2.036	5.40×10^{-04}	0.083
ENSSSCT00000033999	-3.758	61.525	4.335	5.63×10^{-09}	6.58×10^{-05}
ENSSSCG00000001458	-3.762	61.839	4.353	1.39×10^{-08}	3.03×10^{-04}
FAM160A1	-4.807	7.145	0.134	1.72×10^{-05}	0.062
CHST1	-5.418	7.691	0.000	2.16×10^{-05}	0.068

Supplementary Table 4. Ingenuity Pathway Analysis (IPA). List of upstream regulators identified in the set of DEGs according to High and Low myoglobin groups (p-value < 0.05). PAS: Predicted activation state.

Upstream Regulator	Molecule Type	PAS	Activation z-Score	*p*-Value of Overlap	Molecules in Dataset
VCAN	other		-0.928	8.85×10^{-07}	FNDC1,IFI44,IFI44L,IFI6,LGALS3,MX2
IL1RN	cytokine	Low Mb group	2.236	5.78×10^{-06}	IFI44,IFI44L,IFI6,LGALS9,MX2
MAPK1	kinase	Low Mb group	2.646	9.72×10^{-06}	FES,HBA1/HBA2,IFI44,IFI6,LGALS3, MX2,TRIM34
JAK	group			8.30×10^{-05}	CD209,HES6,IFI6
TCR	complex			8.93×10^{-05}	ECH1,IFI44,IFI44L,IFI6,MX2,P2RX7
IFNAR2	transmembrane receptor			9.52×10^{-05}	IFI44,IFI6,MX2
SATB2	transcription regulator			1.16×10^{-04}	EFEMP1,IFI44,MFAP2
IFNA2	cytokine		-1.986	1.18×10^{-04}	EPB41L3,IFI44,IFI44L,IFI6,MX2
IRGM	enzyme			1.39×10^{-04}	IFI44,IFI44L,MX2
LMNA	other			1.56×10^{-04}	ACKR4,COL12A1,EPB41L3,IFI44,PLD 2
oblimersen	biologic drug			1.74×10^{-04}	IFI44,IFI6,MX2
PRL	cytokine	High Mb group	-2.219	2.35×10^{-04}	IFI44,IFI44L,IFI6,MGP,MX2
SPI1	transcription regulator		-1.982	2.63×10^{-04}	CD209,FES,IFI44,IFI44L,IFI6
Interferon alpha	group		-1.047	2.64×10^{-04}	CD209,IFI44,IFI44L,IFI6,LGALS9,MX2
IRF7	transcription regulator	High Mb group	-2.000	3.61×10^{-04}	IFI44,IFI44L,IFI6,MX2
RC3H1	enzyme			3.86×10^{-04}	IFI44,IFI44L,IFI6
CXCL8	cytokine			5.45×10^{-04}	COL12A1,LGALS3,PLD2
IFNL1	cytokine			6.49×10^{-04}	IFI44,IFI44L,IFI6
IRF3	transcription regulator			7.12×10^{-04}	IFI44,IFI44L,IFI6,MFAP2
FAS	transmembrane receptor			8.39×10^{-04}	FES,GTPBP6,LGALS3,MGP,PLD2
PPIF	enzyme			8.67×10^{-04}	ACKR4,CHST1,MGP
TREX1	enzyme			9.32×10^{-04}	IFI44,IFI44L
RNY3	other			1.29×10^{-03}	IFI44,IFI44L
CGAS	enzyme			1.29×10^{-03}	IFI44,IFI44L
CNOT7	transcription regulator			1.70×10^{-03}	IFI44L,IFI6
TLR3	transmembrane receptor			1.78×10^{-03}	IFI44,IFI44L,IFI6,MX2
CLDN7	other			1.82×10^{-03}	IFI44,IFI6,LGALS3
mir-887	microRNA			1.97×10^{-03}	PLD2
MIR3619	microRNA			1.97×10^{-03}	PLD2
NGLY1	enzyme			2.16×10^{-03}	IFI44,IFI44L

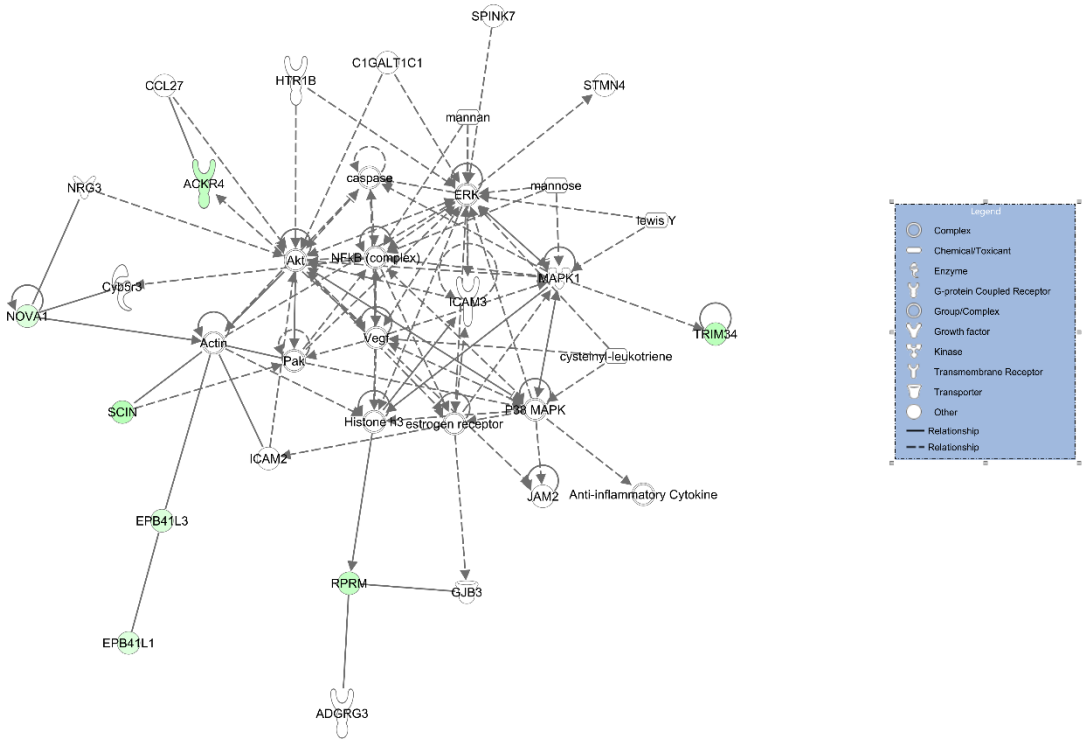
beta-estradiol	chemical - endogenous mammalian	-1.878	2.26×10^{-03}	ACKR2,BICD1,ECH1,EPB41L3,FBLN1,FMO5,HBA1/HBA2,HES6,LGALS3,MGP,PLA2G10,RPRM
PRDM16	transcription regulator		2.28×10^{-03}	IFI44,MX2
Tgf beta	group	-0.762	2.86×10^{-03}	ACKR2,BASP1,CD209,COL12A1
PGR	ligand-dependent nuclear receptor	1.000	2.93×10^{-03}	HBA1/HBA2,IFI44,IFI44L,MX2
mir-96	microRNA		3.10×10^{-03}	IFI44L,IFI6
ZNF106	other		3.40×10^{-03}	HBA1/HBA2,LGALS3
IFNAR1	transmembrane receptor		3.51×10^{-03}	IFI44,IFI6,MX2
stallimycin	biologic drug		3.71×10^{-03}	IFI44,IFI6
TSC2	other		3.77×10^{-03}	IFI44,LGALS3,MGP
CCL14	cytokine		3.93×10^{-03}	ACKR2
HPS1	other		3.93×10^{-03}	LGALS3
ADGRV1	G-protein coupled receptor		3.93×10^{-03}	HBA1/HBA2
ADAMTS13	peptidase		3.93×10^{-03}	LGALS3
COMP	other		3.93×10^{-03}	LGALS3
HBG1	other		3.93×10^{-03}	HBA1/HBA2
CCL3L1	other		3.93×10^{-03}	ACKR2
PAF1	other		4.20×10^{-03}	IFI44,IFI44L
mir-183	microRNA		4.71×10^{-03}	IFI44L,IFI6
IFN Beta	group		5.01×10^{-03}	IFI44,IFI6,MX2
MSC	transcription regulator		5.64×10^{-03}	IFI44,IFI44L
miR-424-3p (miRNAs w/seed AAAACGU)	mature microRNA		5.89×10^{-03}	LGALS3
CYP2C9	enzyme		5.89×10^{-03}	HBA1/HBA2
epiallopregnanolone	chemical - endogenous mammalian		5.89×10^{-03}	LGALS3
ESR1	ligand-dependent nuclear receptor	0.640	5.89×10^{-03}	EFEMP1,FBLN1,HBA1/HBA2,IFI44,IFI44L,IFI6,RPRM,SCIN
POU2AF1	transcription regulator		6.03×10^{-03}	IFI44,IFI44L
IRF1	transcription regulator		6.10×10^{-03}	IFI44,IFI44L,IFI6
STING1	other		6.85×10^{-03}	IFI44,IFI44L
miR-182-5p (and other miRNAs w/seed UUGGCAA)	mature microRNA		7.06×10^{-03}	IFI44L,IFI6
AGT	growth factor	-1.633	7.43×10^{-03}	COL12A1,EFEMP1,LGALS3,MFAP2,NOVA1,PLA2G10

IFNG	cytokine	High Mb group	-2.038	7.49×10^{-03}	CD209,FBLN1,IFI44,IFI44L,IFI6,LGALS3,LGALS9,MX2
CCL17	cytokine			7.85×10^{-03}	ACKR2
FBLN2	other			7.85×10^{-03}	FBLN1
STAT3	transcription regulator			7.91×10^{-03}	CD209,IFI44,IFI6,MX2,PLA2G10
HIPK2	kinase			8.86×10^{-03}	HBA1/HBA2,LGALS3
TLR7	transmembrane receptor			9.02×10^{-03}	IFI44,IFI44L,MX2
Brd4	kinase			9.34×10^{-03}	HBA1/HBA2,LGALS3
MYC	transcription regulator		0.137	9.79×10^{-03}	EFEMP1,IFI44,IFI44L,IFI6,MGP,MX2,SLC16A7
CYP26B1	enzyme			9.80×10^{-03}	P2RX7
SPINDOC	other			9.80×10^{-03}	IFI44L
CCL22	cytokine			9.80×10^{-03}	ACKR2
SPIN1	other			9.80×10^{-03}	IFI44L
TGM2	enzyme			0.0101	IFI6,LGALS9,MGP
poly rI:rC-RNA	biologic drug		-1.982	0.0112	CD209,IFI44,IFI44L,IFI6,LGALS9
PANX1	transporter			0.0117	P2RX7
ZNF395	other			0.0117	IFI44
EFEMP2	other			0.0117	EFEMP1
IL12RB1	transmembrane receptor			0.0117	IFI6
goralatide	chemical - endogenous mammalian			0.0117	LGALS3
SCAP	other			0.0122	HES6,LGALS3
HTT	transcription regulator			0.0129	BASP1,EPB41L3,HBA1/HBA2,MGP,P2RX7
NPC1	transporter			0.013	ACKR4,LGALS3
DRD5	G-protein coupled receptor			0.0137	PLD2
TMPO	other			0.0137	COL12A1
H4-16	other			0.0137	IFI6
RASSF1	other			0.0139	EFEMP1,EPB41L3
mir-122	microRNA			0.0145	BICD1,SH3BGRL3
UBR1	enzyme			0.0156	SLC16A7
ONECUT2	transcription regulator			0.0156	BASP1
Ifn gamma	complex			0.0157	IFI44L,LGALS9
Irgm1	other			0.0157	IFI44,MX2
UBR2	enzyme			0.0176	SLC16A7
TENM1	transmembrane receptor			0.0176	MGP
VWF	other			0.0176	LGALS3
LDB1	transcription regulator			0.019	FBLN1,FES,NOVA1
LMO2	transcription regulator			0.0192	FBLN1,FES,NOVA1

KRAS	enzyme	0.0194	EFEMP1,FES,IFI6,LGALS3,MX2
RNASEH2B	enzyme	0.0195	IFI44
PEA15	transporter	0.0195	PLD2
BTK	kinase	0.0209	IFI44L,MX2
TRIM24	transcription regulator	0.0209	IFI44,LGALS3
CCR2	G-protein coupled receptor	0.0212	COL12A1,FBLN1
LNX2	other	0.0214	HES6
ISG15	other	0.0214	IFI6
CAMK2G	kinase	0.0214	HBA1/HBA2
allopregnanolone	chemical - endogenous mammalian	0.0214	LGALS3
ATP	chemical - endogenous mammalian	0.0223	P2RX7,PLD2
GATA5	transcription regulator	0.0234	HBA1/HBA2
PELP1	other	0.0248	COL12A1,IFI44
SAMHD1	enzyme	0.0253	IFI6
IFNL4	cytokine	0.0253	IFI6
BLM	enzyme	0.0253	IFI44L
DNAJC3	other	0.0272	BASP1
COL9A1	other	0.0272	COL12A1
ATXN7	other	0.0291	BASP1
SERTAD2	transcription regulator	0.0291	ECH1
CAMK2D	kinase	0.031	HBA1/HBA2
Gm12602	other	0.031	BASP1
IRF8	transcription regulator	0.0314	IFI44L,IFI6
SP110	transcription regulator	0.0314	IFI6,P2RX7
SYVN1	transporter	0.0322	IFI44,PLD2
FZD9	G-protein coupled receptor	0.0329	IFI44
MDM4	transcription regulator	0.0329	LGALS3
TOR1A	enzyme	0.0329	BASP1
RARA	ligand-dependent nuclear receptor	0.0347	IFI44,IFI44L,MGP
FMR1	translation regulator	0.0348	BASP1,RPRM
DUSP11	phosphatase	0.0348	IFI44
PKNOX2	transcription regulator	0.0348	RPRM
VGLL3	other	0.0348	COL12A1
GLIS2	transcription regulator	0.0348	MGP

PARP9	enzyme		0.0348	IFI44
TGAL copolymer	biologic drug		0.0348	P2RX7
STAT1	transcription regulator		0.0354	IFI44,IFI44L,IFI6
mir-203	microRNA		0.0367	PLD2
TBXT	transcription regulator		0.0367	MGP
ACTB	other		0.0367	IFI6
APC	enzyme		0.0373	COL12A1,PLA2G10
SMARCD3	transcription regulator		0.0386	MGP
TFAM	transcription regulator		0.0386	ECH1
tretinoin	chemical - endogenous mammalian	-1.294	0.0397	ACKR2,IFI44,IFI44L,IFI6,LGALS9,MGP,P2RX7
HDAC7	transcription regulator		0.0405	RPRM
CCL4	cytokine		0.0405	ACKR2
NR1I2	ligand-dependent nuclear receptor		0.0414	FMO5,MGP
PML	transcription regulator		0.0418	IFI44,IFI44L
MAPK13	kinase		0.0424	EFEMP1
MAPK12	kinase		0.0424	EFEMP1
SFTPD	other		0.0424	CD209
mezerein	chemical - endogenous non-mammalian		0.0424	IFI6
FOXA1	transcription regulator		0.0427	MGP,RPRM
beta-glycerophosphoric acid	chemical - endogenous mammalian		0.0443	MGP
progesterone	chemical - endogenous mammalian		0.0455	COL12A1,FMO5,LGALS3,RAB11FIP5
BMPER	other		0.0462	MGP
DNMT3A	enzyme		0.0465	EFEMP1,P2RX7
RNF20	enzyme		0.0481	HBA1/HBA2
KLF2	transcription regulator		0.0499	FBLN1,LGALS9
CDC73	other		0.0499	LGALS3
ADAMTS12	peptidase		0.0499	MGP

Supplementary Figure 1. Gene network #3: Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking. Genes up-regulated and down-regulated in the High MB group are represented in green and red colors, respectively.



Supplementary Figure 2. Gene network #4: Cardiovascular Disease, Lipid Metabolism, Small Molecule Biochemistry. Genes up-regulated and down-regulated in the High MB group are represented in green and red colors, respectively.