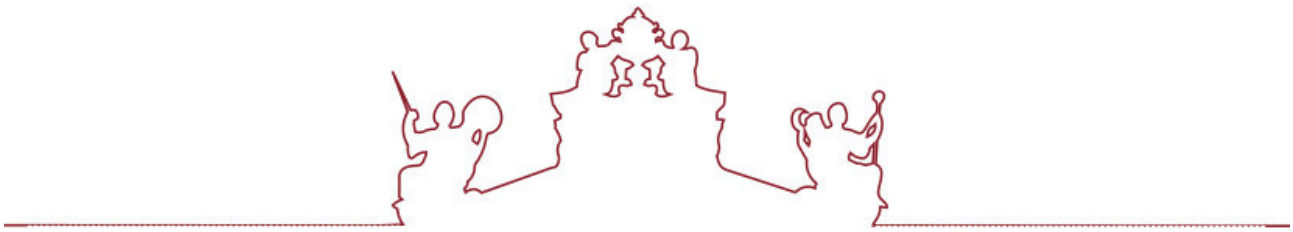

Universidade de Évora - Instituto de Investigação e Formação Avançada
Universidade do Algarve - Faculdade de Ciências e Tecnologia

Programa de Doutoramento em Ciências Agrárias e Ambientais

Tese de Doutoramento

Transcriptomics in Portuguese local pig breeds: an approach to explore the molecular mechanisms underlying lipid metabolism and muscle growth

André Filipe Barreto Albuquerque

Orientador(es) | José Manuel Martins
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The truth is rarely pure and never simple – Oscar Wilde

Experience is symply the name we give our mistakes – Oscar Wilde

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ABSTRACT

This study aimed to characterize the genetic component of economically relevant production traits by exploring the transcriptomics of the dorsal subcutaneous fat (DSF) and *Longissimus lumborum* (LL) muscle tissues from the local Alentejano (AL) and Bísaro (BI) pig breeds using RNA-seq technology and qPCR for validation. DSF yielded 458 differently expressed genes (DEGs), with an overrepresentation of genes associated with *de novo* lipogenesis in AL, agreeing with its fatter profile. Meanwhile, a higher lipolytic activity in BI pigs may be mediated by the growth hormone and AMPK signalling pathways. Lower CD40 signalling in AL suggests lower insulin sensitivity due to the pro-inflammatory effect of the excessive amount of lipids. Higher levels of leptin in AL suggests that this breed has developed leptin-resistance, as previously proposed for the genetically similar Iberian pig. Furthermore, several genes that play important roles in growth were found overexpressed in BI, supporting the idea that development stimuli can occur and be regulated at a multiple tissue level. LL yielded 49 DEGs, with different signalling mechanisms for slow type myosins identified for AL (MYH7) and BI (MYH3), while no markers for fast fibre types were found differently expressed between breeds. On the other hand, the higher expression of *TNNT1* in AL pigs agrees with their lower values of shear force, suggesting the *rigor mortis* process to occur to a less extent in this breed, causing less shortening of the myofibers, lowering the hardiness of its meat. Overexpression of *MAP3K14* in AL pigs may be associated with their lower loin proportion, induced insulin resistance, and increased inflammatory response via NFkB. Finally, overexpression of *RUFY1* in AL may explain their higher IMF content via higher GLUT4 recruitment and consequently higher glucose uptake that can be stored as fat.

Keywords: Alentejano pig, Bísaro pig, lipid metabolism, muscle growth, transcriptome, gene expression.

Transcriptómica em raças suínas portuguesas: uma abordagem para explorar os mecanismos moleculares subjacentes ao metabolismo lipídico e crescimento muscular

RESUMO

Este estudo teve como objetivo caracterizar a componente genética de caracteres com importância económica recorrendo a transcriptómica dos tecidos de gordura subcutânea dorsal (GSD) e *Longissimus lumborum* (LL) das raças suínas Alentejana (AL) e Bísara (BI), utilizando RNA-seq e qPCR para validação. Na GSD foram encontrados 458 genes diferencialmente expressos (GDEs), com uma sobrerrepresentação de genes associados à lipogénese *de novo* em porcos AL, concordando com o seu perfil mais adipogénico. Por outro lado, uma maior atividade lipolítica nos porcos BI pode estar a ser mediada pela sinalização de hormona de crescimento e AMPK. Uma menor sinalização de CD40 em AL sugere reduzida sensibilidade à insulina devido ao efeito pro-inflamatório da excessiva presença de lípidos. Maior expressão de leptina sugere que os porcos AL desenvolveram resistência à leptina, tal como acontece no porco Ibérico. Vários genes encontrados sobreexpressos nos porcos BI corroboram a ideia de que estímulos ao crescimento podem ser regulados em vários tecidos. Apenas 49 GDEs foram encontrados no LL, com diferentes mecanismos de sinalização de miosinas de tipo lento identificados para AL (MYH7) e BI (MYH3). Não foram encontradas quaisquer diferenças entre as raças relativamente a marcadores para fibras musculares rápidas. Por outro lado, uma maior expressão de *TNNT1* em porcos AL concorda com a sua reduzida força de cisalhamento, sugerindo menor influência do processo *rigor mortis*, resultando num menor encurtamento das fibras musculares e menor dureza da sua carne. A sobreexpressão de *MAP3K14* em AL está provavelmente associada à sua menor proporção de lombo e maior resposta inflamatória via NFkB, induzindo resistência à insulina. Finalmente, a sobreexpressão de *RUFY1* em porcos AL poderá explicar o seu maior conteúdo de gordura intramuscular através de um maior recrutamento de GLUT4 que permite uma maior absorção de glucose, a qual pode ser convertida em gordura.

Palavras-chave: porco Alentejano, porco Bísaro, metabolismo lipídico, desenvolvimento muscular, transcriptoma, expressão genética.

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LIST OF ABBREVIATIONS

ACACA	Acetyl CoA carboxylase
ACEPA	Alentejano Pig Complementary Consortium of Companies
ACLY	ATP citrate lyase
ACPA	Association of the Alentejano Pig Breeders
ACTB	Actin beta
ADG	Average daily gain
ADIPOQ	Adiponectin
ADIPOR	Adiponectin receptor
AL	Alentejano
ALAS1	5'-Aminolevulinate synthase 1
AMPK	Adenosine monophosphate-activated protein kinase
ANCPA	National Association of the Alentejano Pig Breeders
ANOVA	Analysis of variance
APOD	Apolipoprotein D
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
ATP6V1C1	ATPase H+ transporting V1 subunit C1
BI	Bísaro
BLUP	Best linear unbiased prediction
BMPs	Bone Morphogenetic Proteins
Bp	Base pairs
BIRC7	Baculoviral inhibitor of apoptosis protein repeat containing 7
BW	Body weight
CBX1	Chromobox 1
CCAAT	Cytosine-cytosine-adenosine-adenosine-thymidine
CCC	Concordance correlation coefficient
CCL2	C-C Motif chemokine ligand 2
cDNA	Complementary desoxyribonucleic acid
CEBPA	CCAAT/enhancer-binding protein alpha
CEBPB	CCAAT/enhancer-binding protein beta
CEBPD	CCAAT/enhancer-binding protein delta
CIC	Citrate carrier
CNNM3	Cyclin and CBS domain divalent metal cation transport mediator 3
CoA	Coenzyme A
CPT1	Carnitine acyltransferase 1
CPT2	Carnitine acyltransferase 2
CSNK1D	Casein kinase 1 delta
CT	Cycle threshold
CTSF	Cathepsin F
DAVID	Database for annotation, visualization, and integrated discovery

DEGs	Differentially expressed genes
DSF	Dorsal subcutaneous fat
DUSP1	Dual specificity phosphatase 1
EGR1	Early growth response 1
ELN	Elastin
ELOVL6	Elongation of long-chain fatty acids family member 6
ERK	Extracellular signal-regulated protein kinase
FA	Fatty acid
FABP4	Fatty acid binding protein 4
FAD	Flavin adenine dinucleotide
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization
FASN	Fatty acid synthase
FBXO32	F-Box protein 32
FC	Fold change
FDR	False discovery rate
FGF	Fibroblast growth factor
FOS	Fos proto-oncogene, AP-1 transcription factor subunit
FOSB	FosB proto-oncogene, AP-1 transcription factor subunit
FOXO1	Forkhead box O1
GC	Guanine-cytosine
GC-MS	Gas chromatography-mass spectrometry
GEO	Gene expression omnibus
GH	Growth hormone
GLUT4	Glucose transporter 4
GTPase	Guanosine triphosphate hydrolase
GWAS	Genome wide association study
HOTAIR	Homeobox transcript antisense intergenic RNA
HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase
HMGCS	3-Hydroxy-3-methylglutaryl-CoA synthase
HSPCB	Heat shock protein 90 alpha family class B member 1
IB	Iberian
IGF	Insulin-like growth factor
IL	Interleukin
IMF	Intramuscular fat
INSIG	Insulin induced gene
INTS11	Integrator complex subunit 11
IPA	Ingenuity pathway analysis
IRF2	Interferon regulatory factor 2
KDM2B	Lysine demethylase 2B
KLB	Klotho beta
KLF	Kruppel like factor
LDL-c	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LEP	Leptin
Leu	Leucine
LL	Longissimus lumborum

LMOD	Leiomodin
LPL	Lipoprotein lipase
LSP1	Lymphocyte specific protein 1
MAFbx	Muscle atrophy F-box protein
MAGL	Monoacylglycerol lipase
MAP3K14	Mitogen-activated protein kinase kinase kinase 14
MDH	Malate dehydrogenase
ME1	Malic enzyme 1
MED1	Mediator complex subunit 1
MEF2C	Myocyte enhancer factor 2C
Met	Methionine
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
MUFA	Monounsaturated fatty acid
MVK	Mevalonate kinase
MYD88	Myeloid differentiation primary response 88
MYH	Myosin heavy chain
MYOD	Myogenic differentiation
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National center for biotechnology information
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-kB inducing kinase
NGS	Next-Generation Sequencing
NR1D1	Nuclear receptor subfamily 1 group D member 1
P4HB	Prolyl 4-hydroxylase subunit beta
PCK1	Phosphoenolpyruvate carboxykinase 1
PCR	Polymerase chain reaction
PDGFB	Platelet derived growth factor subunit B
PDO	Protected Designation of Origin
PGI	Protected Geographical Indication
PI3K	Phosphoinositide 3-kinase
PPARA	Peroxisome proliferator activated receptor alpha
PPARG	Peroxisome proliferator activated receptor gamma
PSE	Pale, soft and exudative
PUFA	Polyunsaturated fatty acid
PTGES2	Prostaglandin E synthase 2
qPCR	Quantitative PCR
QTL	Quantitative trait locus
RABEP2	Rabaptin, RAB GTPase binding effector protein 2
RABIP4	RUN and FYVE domain containing 1
RELA	RELA proto-oncogene, NF-kB subunit
RI	Ribatejano
RIN	RNA integrity number
RNA-seq	Ribonucleic acid sequencing
RPL19	Ribosomal protein L19
rRNA	Ribosomal RNA
rt-PCR	Reverse transcription polymerase chain reaction

RT-qPCR	Real time-quantitative PCR
RUFY1	RUN and FYVE domain containing 1
SCAP	SREBF chaperone
SCD	Stearoyl-CoA desaturase
SE	Standard error
SEPTIN1	Septin 1
Ser	Serine
SFAs	Saturated fatty acids
SINE	Short interspersed nuclear elements
SMYD5	SET and MYND domain-containing protein 5
SNPs	Single Nucleotide Polymorphisms
SPSB2	SplA/ryanodine receptor domain and SOCS box containing 2
SREBF	Sterol regulatory element-binding transcription factor
SREBP1-c	Sterol regulatory element-binding protein 1c
SRF	Serum response factor
STAT3	Signal transducer and activator of transcription 3
STMN3	Stathmin 3
TAS2R39	Taste receptor type 2 member 39
THRA	Thyroid hormone receptor alpha
TNF	Tumor necrosis factor
TOP2B	DNA topoisomerase II beta
TR/RXR	Thyroid hormone receptor of the nuclear retinoid X family
TNNT1	Troponin T1
UCP1	Uncoupling protein 1
V-ATPase	Vacuolar-type proton-translocating ATP hydrolase
WBSF	Warner-Bratzer shear force
WDR91	WD Repeat Domain 91

Chapter 1

General Introduction

Pigs and humans share a unique and close relationship that dates to over 10 000 years ago. Today's domesticated pigs are commonly acknowledged for their intelligence, fertility, voracious appetite and, probably undeservedly, their sloppy and dirty nature. Contrarily, their ancestor's wild boar has always been associated with a fierce and courageous nature, feared by humans through the ages. These wild beasts were a much important prey for Eurasian hunter-gatherer societies during the Palaeolithic age, particularly before agricultural insurgency, as suggested by genetic and archaeological evidence (Groenen et al., 2012). The first Agricultural revolution, a crucial step in human history, allowed for communities to settle and experiment with plants and animals, leading to the domestication of the first animals, which firstly promoted the shift in wild boars to what we currently recognize as the domestic pig (Albarella et al., 2007).

Pigs have endured several other anthropogenic influencing processes over the ages besides hunting, and these included deforestation and translocation of populations. These processes strongly shaped population numbers and, ultimately, lead to biodiversity loss. More recently, intensive production of pig meat and its derived products have also caused losses regarding genetic diversity since intensive pork production systems mostly rely on producing breeds with the best commercial value (Frantz et al., 2016). Traditionally, ancient European local breeds, well adapted to their environments, were rarely selected. These animals were fed fruits of woody plant species, accumulated on forests grounds. As industrialization and capitalism emerged in early modern European societies and forests were increasingly cleared, feeding with nuts or fruits of trees and shrubs (such as beechnuts, acorns, and berries) was no longer sufficient to keep up with demand and import of highly productive Chinese pig types played a role in developing local breeds into improved breeds and varieties. These new breeds and varieties, focused on accelerated weight gain and higher feed conversion rates, transformed pig farms from small-scale subsistence-oriented farms to industrial meat production machines. Moreover, intensification of agricultural systems based on massive land exploitation, and industrialization of pig farming, together with the overcoming predominance of these high production breeds, have reduced the relevance and population numbers of native breeds (Pugliese and Sirtori, 2012; White, 2011).

Nowadays, pigs continue to be raised mostly to satisfy meat-producing industries to keep up with consumers' demands. World pork requirements have been continuously increasing, mostly due to adjustments in the consumption patterns of developing countries with fast-growing economies. In this current context of expanded international trade and profitable projects, breeders' choice when investing in livestock production is influenced by several factors including market trends, competition, price consistency and trade restrictions. Contrarily, small-scale farmers commonly struggle to acknowledge and respond to new challenges and take advantage of market opportunities, failing to keep up with the competition posed by industrial producers. Furthermore, the recent developments in pig breeding technologies have transformed pig production into a high-input, high-output commercial industry, focused on exploiting the full potential of a small number of selected highly productive breeds in standardized environments. Local pig breeds are usually overlooked since they cannot be

competitive in the concept of modern pig production, which poses a challenge in preserving these genetic resources. A low population size also poses a threat of higher inbreeding rates within local pig breeds, which are already undermined by their generally lower litter sizes. During the 20th century, numerous breeds severely declined in population and some even became extinct. These events led to the development of policies and breed conservation programs to effectively respond to the loss of biodiversity observed (Čandek-Potokar et al., 2019; FAO, 2015). Nowadays, small-scale farmers still rely on local breeds, well adapted to their environments, or crosses with them to supply pork and quality dry cured products to niche markets. These breeds differ from improved ones regarding several meat quality traits, namely the amount of intramuscular fat (IMF) content, which is generally higher in local breeds and boosts the organoleptic properties of their meat. Furthermore, local pig production chains agree with the concerns of modern consumers about animal welfare and low environmental impact, all while promoting local pure breed populations providing high quality meat and meat products (FAO, 2015; Martins et al., 2020; Pugliese and Sirtori, 2012).

For more than 30 years, the numerous similarities between humans and pigs have also prompted the ability to use them as a human biomedical model, boosted by the availability of the complete swine genome (Walters et al., 2012). Employment of animal models to simulate human conditions has been a common practice since long, providing a chance to better study them while pursuing improved strategies to address them. For decades, rodents have been the obvious choice due to their low maintenance requirements, their easiness to handle and rapid breeding profile. Nevertheless, rodent models cannot adequately replicate specific human diseases such as lateral sclerosis, Alzheimer's, Parkinson's, and many types of cancer. Pigs, on the other hand, have been highly included in models for translational research, showing incredible potential regarding xenotransplantation, mostly due to their anatomical and physiological similarities to humans, such as organ size and function, and disease progression (Walters et al., 2017; Walters et al., 2012). These similarities, however, do not translate to a closer genetic ancestry between humans and pigs, but are rather attributed to a fortuitous convergent evolution, where similar selective environmental pressure promoted the development of similar physical traits. Curiously, despite rodents' genetic sequence being closer to humans than the one of pigs, other genetic elements besides gene composition have proven that pigs are more closely related to primates than what has been previously though. For example, the discovery in pigs of a unique family of short interspersed nuclear elements (SINEs), whose evolution has closely paralleled humans, might have uncovered a hidden evolutionary link between both species (Yu et al., 2015).

A 2020 report by the Portuguese Federation of pig farmers associations (FPAS, "Federação Portuguesa de Associações de Suinicultores") stated that a population of 197 932 reproductive pigs were registered (FPAS, 2020) in Portugal, from which 5.2% of these were of pure Portuguese local breeds (ANCPA, 2022; ANCSUB, 2022b; CAP and DGAV, 2021). Local pig breeds are found less competitive when compared to modern, intensively selected ones, particularly in terms of productivity. Unlike other pig breeds, Portuguese Alentejano (AL) and Bísaro (BI) breeds were not

yet submitted to a systematic selection for productive traits and, therefore, any development is primarily related to empirical selection performed by farmers. Local pig breeds such as the AL and BI represent a pool of ancient genetic resources and a link to traditional production systems and pork products that needs to be valued. These and other local breeds and their production systems struggled for survival in the past but have recently grown in interest within the scientific community and general population. Exploring the genetic diversity within these breeds will help better understanding of the underlying physiological mechanisms associated with body fat deposition and how they influence meat quality. In this work, AL and BI pigs were selected for phenotypical characterization which included, among other parameters: colour determination using a Minolta colorimeter instrument (*L*a*b** system), ultimate pH, total protein quantification, myoglobin content, total and soluble collagen contents, and lipid content and composition.

The main goal of this thesis was to obtain new molecular data using recent transcriptomic strategies, including ribonucleic acid sequencing (RNA-seq) and Real-time quantitative polymerase chain reaction (RT-qPCR), providing new knowledge about the genetic component of phenotypical traits to improve existing breeding programs, or develop new ones, for the local AL and BI pig breeds.

The specific goals of this thesis were the following:

- Summarily describe the phenotypical traits of adult AL and BI pigs (approximately 150 kg BW), the two most important local pig breeds from Portugal;

- Obtain the total RNA and sequence, through Next-Generation Sequencing (NGS) technologies, the complete transcriptome of dorsal subcutaneous fat and *Longissimus lumborum* samples from purebred AL and BI pigs;

- Assess which genes display contrasting expression patterns within these breeds using a differential expression analysis and confirm the results using RT-qPCR;

- Associate the identified differentially expressed genes (DEGs) with the determined phenotypical data to explain the differences between the breeds and proclaim their intrinsic genetic value.

1.1. Historical perspective and phenotypical traits of Portuguese pig breeds

1.1.1. The Alentejano breed

Currently, three local pig breeds are officially inscribed within the catalogue of Portuguese breeds, out of a total of 61 breeds across all species (livestock, poultry, and dogs). From these, the breed historically most represented in Portugal, is the AL pig, currently with 317 males and 4539 females, distributed among 111 farms (data registered at the end of 2021 in the breed's Herdbook, ANCPA (2022)). AL pigs are a great example of an animal well adapted to their environment. This breed is mostly raised in the southern part of Portugal (Alentejo region, south of the Tagus River) mainly in extensive/semi-extensive production systems, in a region characterized by extensive areas of low slopes, poor and highly degraded soils, irregular climatic conditions and where the "Montado" ecosystem is predominant. This system is mainly composed by oak forests which allows for AL pigs to take full advantage of the natural resources available including acorns and grass as feed, while enduring periods of lower feeding availability due to a high capacity to accumulate and store fat (CAP and DGAV, 2021; Charneca et al., 2019).

AL pigs evolved from the primitive *Sus scrofa mediterraneus*, which settled in the Iberian Peninsula around 6000 B.C. and originated the Iberian genetic lineage that includes other famed Spanish varieties such as the Entrepelado, Retinto, Lampiño, and Torbiscal, among others (Gama et al., 2013). The closest genetic relatives to these breeds include the Negre Mallorquí (Majorcan black) native to the Balearic Islands, the Hungarian Mangalitsa and the Italian Mora Romagnola (Muñoz et al., 2018). Historical references from the Roman Empire, during the first two centuries A.D., mention AL and Iberian (IB) pigs for their adaptability to outdoor rearing in holm oak forests and their renowned meat products (Daza, 1996; Janeiro, 1944). Later during the Medieval era, with the arrival of the Arabians to the Iberian Peninsula, Iberian pig populations were predicted to decrease due to Arabic religious beliefs against pork consumption. However, these predictions did not come true, and pork consumption even increased during this period, with pig meat being considered a very healthy food source and even a social status marker (CAP and DGAV, 2021; Grau-Sologestoa, 2017).

Until the beginning of second half of the 20th century, this unselected pig breed continued to be the most economically important and representative in Portugal, attaining over 45% of the total pig population. Pigs were raised traditionally, with the later fattening period called "montanheira" (comparable to the Spanish "montanera") occurring each year between the end of October until February. Slaughtering occurred when pigs achieved 120-150 kg (18-24 months), with their meat being mainly used for the production of high quality dry cured meat products (Carvalho, 1964; Freitas et al., 2004; Freitas et al., 2007). In the early 1950s, it was estimated that over 400 000 AL pigs existed in the districts of Évora, Beja and Portalegre. AL pig production was

of major importance for local industrial and commercial development but had also an impact at national level. Furthermore, the AL breed blends with the historical and cultural heritage of Portugal and plays an important role in the settlement of population, particularly in rural areas. These pigs supplied local meat demands as well as slaughterhouses, and artisanal meat processing industries dedicated to the production of dry-cured or dry-fermented meat products such as "presunto", "linguiça", "chouriço" and "paio". The remaining animals supplied meat for Lisbon and other meat processing industries near the capital (Freitas et al., 2004).

In Europe, animal production evolved substantially after the Second World War. Genetic improvement, advances in animal sanitation, generalized use of standardized feeds, major investments on facilities and an increased intensification of the production systems, provided significant productivity boosts. During the following decades (1950-1990), AL pigs suffered a severe decline in population numbers, namely due to the generalized intensification of pig production systems and their logical replacement with leaner, more productive breeds, focused on rapid meat production. On a consumer's perspective, a shift in eating habits occurred towards eating meat with less fat, and vegetable fat sources were preferred to animal ones. The political guidelines favoured intensive swine production, without the same land requirements of extensive systems. In addition, the increased mechanization of agricultural practices, along with the outbreaks of African swine fever, led to the downfall use of the "montanheira" system and, consequently, a reduction in the number of AL pigs. In the 1980s, this breed was even on the brink of extinction. Most of the remaining animals were used to cross with leaner industrial types to sell weaned piglets, while a reduced interest for AL pigs, particularly the ones grown in the "montanheira" system, was observed (Frazão, 1984; Freitas et al., 2004). The replacement rate of reproducing males and females per herd was very low and inbreeding was, on the other hand, quite elevated (Correia, 1989).

The risk of extinction has been, however, prevented with European and national policies involving the application of communitarian aids, along with the efforts of pig producers and their newly founded organizations such as UNIAPRA ("União das Associações de Criadores do Porco Raça Alentejana") which merged the Association of Alentejano pig Breeders (ACPA) with the National Association of the Alentejano Pig Breeders (ANCPA). This new association succeeded in uniting efforts from both associations, from south and north of Alentejo, respectively, and was designated administrator of the Portuguese Genealogical Book of Pigs – Alentejano Breed section. In the early 2000s, UNIAPRA joined with the Spanish Association of Iberian pig breeders, AECERIBER, and the French "Consortium du Noir de Bigorre" (Gascon pig), to create the *Federación de Razas Porcinas Autóctonas y Extensivas de la Unión Europea*, FESERPAE. This new Association had the purpose of protecting the interests of South European local extensive pig breeds, as well as promoting synergies regarding technical and scientifical capabilities towards mutual development. Additionally, the late 1980s brought into attention the pollution issues caused by intensive pig production systems, and their progressive worldwide environmental impact, which posed new handicaps for producers and governors. Meat and meat products quality, as well as animal welfare also became more relevant, accompanying a new dynamic

which diversified the saturated pig meat market, and developed a renewed interest in alternative production systems (Covas, 1993; Freitas et al., 2004). This interest grew even more during the 1990s and can be associated with the elevated pack of amends launched in 1992 by the European Agricultural Common Policy. These amends were focused on the accountability of famers towards environmental protection and sustainable agriculture, in order to preserve biodiversity and natural landscapes, and fight climate change, while increasing production to satisfy the predictable rise in feeding demands in the EU (Freitas et al., 2006b; Qureshi, 1993).

Today, the population of AL pigs is more stable. These animals are mainly used for the manufacture of quality products, such as sausages and hams, that characterize and enrich the regional gastronomy, and to sell for the fresh meat market as high-quality meat, rich in oleic acid and offering a premium source of protein. However, the AL pig is still classified as an endangered Portuguese breed, classified with a moderate risk of extinction in 2015 by the Portuguese Ministry of Agriculture and Sea (https://dre.pt/web/guest/pesquisa/-/search/66619894/details/maximized), leading to financial support of the producers, through European funds, to preserve the genetic resources of local breeds at risk (Charneca et al., 2019).

The external phenotypical traits are related to AL's extensive production system, where pigs are encouraged to explore vast plain fields in search for food. AL pigs are generally characterized by their medium to small size, light bone structure and dark skin, sometimes with the presence of black, blonde, or red bristles of very thin hair (Figure 1). The head is long and thin, where we can also find a distinct, not very sharp, frontonasal angle, and the triangularly shaped small and thin ears, inclined to the front, with the tips slightly shifting outside. The neck is muscled and of average length. The back is slightly arched, and of medium size, as are the shoulders and hams. The medium to small limbs are lean and their short feet have strong black hooves. Tails are thin and of medium insertion, and the females commonly present a minimum of 10 teats while the males display bulging, averagely voluminous testicles (CAP and DGAV, 2021; Charneca et al., 2019).

Figure 1. Example of an Alentejano sow with piglets (Adapted from Charneca et al. (2019)).

AL pigs present a great level of rusticity and an energetic nature. Four varieties of AL pigs can be distinguished: Lampinha, Ervideira, Caldeira and Mamilada. The Lampinha variety is characterized by its black to grey colour, rarely found bristles, their big and sagging ears and for presenting a lower overall development, when compared to the other varieties. The Ervideira variety colour can range from clear red to brownish, with the head and ears slightly smaller than the black varieties, and its major differentiating feature being the presence of multiple depigmented zones in the hooves. The Caldeira variety vary between the reddish and black colours, but no depigmented zones are observed. Finally, the Mamilada variety is characterized by a colour ranging from black to red, with rare thin bristles, and an underdeveloped jowl where the presence of nipples is a distinct feature. Nevertheless, due to the drastic reduction in population observed in the past century and the continued crossing between them and with other breeds, the prevalence of all these varieties today is many times questioned. In fact, since the 40s it has been observed, for example, that Ervideira pigs were being replaced with the darker variety Caldeira. (CAP and DGAV, 2021; Carbó and Andrada, 2001; Frazão, 1965).

As stated before, AL pigs are well adapted to the typical “Montado” landscape, of the Mediterranean region, packed with *Quercus* forests. In fact, the late fattening period “montanheira” is a strategic period of the traditional production system, in which the animals feed of the available acorns and grass, usually between the months of October and February.

Traditionally, AL pig production was based on a strategy where the animals were divided into three categories: breeding sows, growing pigs, and fattening pigs. Breeding sows had two litters per year, the first during spring (between March and May), and the second during the fall season (between September and November). Breeding sows and growing pigs were mostly fed with natural pastures and, when necessary, supplemented with cereal grain (oats, corn or barley), legume crops (chickling vetch, faba bean or black chickpea) or other local agricultural by-products, which would limit growth and increase the overall extent of the productive cycle. Piglets were fed with maternal milk until 20-25 days old and at 30-45 days of age would start to go to pastures and be supplemented with some barley grain. Weaning would occur at about 2 months of age, and the castration of the piglets destined to fattening would occur at around 3 months (Frazão, 1965; Pereira, 1945; Póvoas Janeiro, 1951). "Montanheira" for fattening pigs would be the last step of the production system, with pigs feeding on acorns and available pasture. This system worked with the purpose of minimizing production costs, so the animals had to survive while managing the least possible cost for the producers, which lead to periods of hunger, particularly between the cereal leftovers consumption of summer and the starting of the "montanheira" period during fall. Therefore, in many farms with lower agricultural resources available, the period between August and October would involve animal supplementation with commercial feeds to avoid a dramatic loss of weight. This period contrasted with the following "montanheira", where feeding and daily weight gain were very high. The fattening period in "montanheira" is one of the key factors to obtain better raw materials that will be transformed in high-quality meat products (Fernandes et al., 2008; Frazão, 1965; Freitas, 2014; Freitas et al., 1995).

Nowadays there is no common production system applied to AL pigs. Timing for reproduction, feeding management, and weight and age for slaughter change between different producers, depending on the goals of the production system and tradition issues (Charneca et al., 2019; Freitas, 2014). The most used production systems remain the extensive and semi-extensive systems, with the existence of an outdoor feeding period as mandatory for the production of PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication) products. Nevertheless, growing AL pigs using semi-extensive systems is continuously increasing in popularity, due to the improved and homogenized animal performance and productivity, with growing and fattening pigs being fed commercial feeds, specially developed for their nutritional needs. Most herds still rely on two litters per year, the main one occurring between October and December, and the other from April to June. Piglets born from the main litter will be used in "montanheira" in the next year, while the others will be used to replace adult pigs, and to supply the market of roasted piglets, of fresh meat, and sausage industries (Freitas, 2014; Freitas et al., 1995; Freitas et al., 2006b). Sometimes, as it happens in Spain with the IB pig, this last litter is the result of crossing AL females with Duroc boars, to obtain crossed pigs with better growing yield and performance, as well as leaner carcasses. Piglets are fed with maternal milk until weaning, however sometimes supplementation with commercial feeds may be required. Weaning generally occurs when piglets are 45 to 60 days old and 10-14 kg (BW). This late weaning is

justified by the low growth rates observed in AL piglets and the absence, in most herds, of adequate environmentally controlled facilities. After this, the growing period will extend until the beginning of the fattening period, including a post-weaning period between 14-25 kg BW. The fattening period begins when pigs attain about 100 kg BW. The average daily gain (ADG) during the lactation, the growing and fattening periods are lower than the ones observed in modern leaner breeds, varying between 142 and 191 g/day during lactation to a maximum of 1000 g/day during "montanheira". Despite ADG increases with age and a major increase during the fattening period, overall rearing of AL pigs is also characterized by high heterogeneity. The higher variability could be associated with differences associated to the rearing systems implemented by the different producers, and/or the environmental conditions. Nevertheless, approximately 1000 g/day of ADG should represent a fair estimation of the maximum growth potential displayed by AL pigs at *ad libitum* feeding conditions (Charneca et al., 2019).

The average BW of the adult AL pigs can attain about 160 kg in boars, and 120 kg in sows. The age or weight at which the Alentejano pigs are slaughtered will depend on the market/industry to supply: roasted piglet market, meat for fresh consumption, raw material for the sausage industry, and raw material for the ham industry in the Portuguese or the Spanish market (Charneca et al., 2019; Freitas, 2014). Table 1 summarizes the most common practiced slaughter weights and ages for AL pigs.

Table 1. Common slaughter weights and ages for Alentejano pigs and their respective target markets.

Slaughter weight	Slaughter age	Target market
10-14 kg	45-60 days	Roasted piglets
90-100 kg	8-14 months	Fresh meat
120-140 kg	14-20 months	Sausage industry
150-170 kg	14-24 months	Ham industry

The feed resources available within each farm limit animal feeding, particularly during the fattening period, which can exclusively occur in "montanheira" or in a mixed regimen, with food provided by nature (including grass, acorns, and cereals, legumes and other crops by-products), a complementary diet based of cereals produced in the farm (including corn, barley, and triticale) and alternative feeds, either produced in the farm or bought directly from the market (Freitas, 2014).

Compared to the traditional system, the main changes introduced in the current production systems imply, for example, the use of facilities and production methods generally associated with more intensive systems. These include animal management in groups, earlier weaning, more efficient sanitation programs and new feeding strategies to better respond to the

different periods of the animal life cycle. In this last issue, it should be highlighted the importance of composed feeds in balancing feeding regimens, and the role of the feed industry in developing quality feeds, specifically for AL pigs reared in the extensive system (Fernandes et al., 2008; Freitas et al., 2006b). These changes have been responsible for the shortening of the production cycle, while making it a more efficient process, usually without damaging the properties and overall quality of the obtained products.

Most of the times AL sows are naturally mated, although artificial insemination is a possibility in the market. Females start their reproductive life after the first year of age and present, normally, low prolificacy and milk production. Table 2 summarizes various reproductive traits of AL pigs (Charneca et al., 2019). The gestation period is often shorter than in other breeds, and litters present an average between seven to nine piglets alive. The higher range of stillborn per litter is often related with higher prolific females, therefore, the number of AL stillborn piglets is generally lower than the ones reported in other, modern breeds with high prolificacy (Canario et al., 2007). On the other hand, the high death rates at weaning agree with what is generally observed in other Iberian pig types (González et al., 2007). Due to the low growth rates during lactation of AL piglets and the conditions after weaning, lactations are generally longer than the ones observed at more intensive systems, lasting between 35 to 60 days, which leads to an increase of the farrowing interval and reduce overall productivity (Freitas et al., 2006a; Marques, 2001).

Table 2. Summary of the main reproductive parameters of Alentejano pigs (Adapted from Charneca et al. (2019)).

Parameter		Reference
Age at first parturition (months)	10.6-16.6	ǂ, ¥
Litters per sow per year	1.8	ǂ
Piglets alive per litter	6.7-9.4	(Nunes, 1993)
Live weight at birth	1.0-1.3	(Póvoas Janeiro, 1951)
Stillborn per litter (%)	1.2-11.3	(Charneca et al., 2012)
Mortality at weaning (%)	18.8-27.0	(AGRO_254, 2007; Fernandes et al., 2008)
Gestation period (days)	111	(Charneca et al., 2012)
Farrowing interval (days)	206	¥

Notes: ǂ Charneca, unpublished work, Universidade de Évora, 2016; ¥ ANCPA, Personal communication, database including 20 farms.

1.1.2. The Bísaro breed

The historically second most important local pig breed in Portugal is the BI, currently with a total of 421 males and 5045 females, distributed amongst 144 breeders (data registered in the end of 2021, in the breed's Herdbook, (ANCSUB, 2022b)). BI pigs have been a biological,

economic, and cultural heritage for centuries and a valuable resource for local communities, mainly due to their much-appreciated products such as smoked sausages. This breed is mostly raised within the northern regions of Portugal (north of the Tagus River), following an open air intensive or extensive production system (Paixão et al., 2018). Several reasons are pointed for the survival of this breed until present day, including their good adaptation to the environment, their gentleness, high prolificacy and great meat quality traits that allow the production of high-quality regional products (CAP and DGAV, 2021; Santos Silva et al., 2019).

Tracing back to its earlier origins, BI pigs descend from the domestication of the European boar, *Sus scrofa ferus*, a process that resulted in the development of the Celtic breed group (Janeiro, 1944). BI pigs were raised throughout the North and Centre of Portugal until half of the 20th century. Similar to what happened with breeds of the Mediterranean Iberian lineage, the Celtic lineage almost became extinct in the past century due to heavy animal crossbreeding with foreign pigs from Asia and the African swine fever outbreaks between the 1950s and 1970s, among other factors (Santos Silva et al., 2019; Vieira and Ramos, 1985). Fortunately, conservation and recovery programs of BI populations started in the 1990s, brought the overall number of sows and boars to more stable, resilient values (Santos Silva et al., 2019).

During the 19th century, Macedo Pinto (Pinto, 1878) classified them as Bísaro type 1 or Celtic, to express the ancientness of these pigs, as he believed they were a variation of a Celtic Pig from ancient Gaul. Some genetic relatives to this breed include other Celtic types, natives to several Spanish regions such as the Celta pig from Galicia, the Chato Murciano from Murcia, the Negro Canario from the Canary Islands and the least representative Portuguese local pig breed, the Malhado de Alcobaça (MAL) (Gama et al., 2013). Contrarily to what is assumed about the Mediterranean group regarding a prior and fixed presence in the Iberian Peninsula, Celtic pigs like BI are believed to descend from northern-central European pigs. There are also suggestions of the introduction of Chinese germplasm in a distant past (Gama et al., 2013; Royo et al., 2007). Therefore, it is not surprising that the genetic distance between Celtic and wild types is generally much higher when compared to the one between Mediterranean and wild types, which seems to confirm higher mixing between the latter two throughout the ages. Furthermore, the large genetic distance and separated cluster formation observed between Celtic and Mediterranean groups illustrate the distinct breeding patterns between both groups (Gama et al., 2013).

BI pig production dominated in the North of Portugal until the 50s of the 20th century. Traditional production systems and management of this breed evolved according to the local agricultural practices and the available resources of agroforestry by-products in the region. By the end of the 19th century, Eurasian swine breeds were introduced for the first time in Portugal. During the 60s and 70s of the 20th century a few crossbreeding attempts between local and exotic pig breeds were performed, a tendency that increased later with massive import of foreign leaner breeds. Consequently, and similar to what happened to AL pigs during the same period of time, these events together with the already mentioned higher popularity of intensive production systems, focused on rapid meat production, accelerated the disinterest towards local breeds and lead to its genetic erosion (Cordeiro, 1988). In the 1990s, BI pigs were almost extinct, limited to

about 100 individuals in small-scale farms at the North of Portugal. Conservation projects and recovery programs introduced by the government afterwards, together with the creation of the National Association for Bísaro Pig Breeders (ANCSUB) in 1994, helped to increase the overall numbers of this breed. These programs also allowed for an extensive characterization and assisted the producers with new knowledge to better develop the BI breed, currently a work in progress (Santos Silva et al., 2019). Despite the positive results, several BI populations still struggle with high inbreeding levels and low number of individuals per farm (Paixão et al., 2018). In fact, management of reproductive cycles is frequently disregarded, and only a minority of producers seek artificial insemination as a reproductive method, aspects that contribute to higher inbreeding risks. A recent study (Paixão et al., 2019) mentioned the large genetic potential that is not currently being explored in BI pigs selection programs and that even despite lower heritabilities in most reproductive traits, the moderate variance components obtained indicate that these traits might respond well to more efficient selection programs.

Morphologically, Bísaro pigs are characterized by their high corpulence and strong bone structure, the body attaining up to 1 m in height and 1.8 m from the nape to tail insertion. Hair colour can vary between black, grey, white, or spotted and the skin is thin with large and abundant bristles (Figure 2). The head is large (40-45 cm length), presenting a long and concave snout, while the ears are floppy and extra-large, usually covering the eyes. The neck is long and muscular, while the back is long in length and convex in shape. The abdomen is thin, as are the thighs and the long legs. The feet are well developed, and the tails (55-60 cm length) are thick and of average insertion. Less rustic and agile than AL, the BI breed has a docile temperament and is well adapted to the traditional system. BI sows frequently present a minimum of twelve working teats (CAP and DGAV, 2021; Santos Silva et al., 2019).

Figure 2. Example of a Bísaro sow with piglets (Adapted from ANCSUB (2022b)).

A total of two BI varieties can be distinguished: the first, Galega, is characterized by white or white-spotted colour patterns, common in the Minho region; the second, Beiroa, present black or black spotted colour patterns and is commonly raised in the regions of Beira, Trás-os-Montes, and Minho. Both varieties have long and hard bristles. A third variety, Molarinhos, has been recently declared extinct and was characterized by the absence of bristles (CAP and DGAV, 2021; Santos Silva et al., 2019).

The overall intensification of BI production systems within their corresponding geographical area of origin as fluctuated through time. Traditionally, in the 20th century, BI pigs were grown in a system called "vezeira" based on large outdoor areas for feeding. The animals would cross extensive land areas in search for available grass and acorns, produced by the existent oak trees (ANCSUB, 2022b; Santos Silva et al., 2019). In familiar small-scale farms there were commonly one or two BI sows, because pigs were seen as an economic supplement for their own subsistence. This was obtained through direct consumption or for processing artisanal products, sold directly to consumers or during local small fairs. In this production system, most of the times the available forest resources would be scarce and fail to fully feed the farm animals which would lead to the use of agricultural products and by-products (Santos e Silva and Nunes, 2013). These complementary food supplies would include cereals (corn, wheat, triticale, bran, and barley), forage feeds such as tubers (potatoes, turnips, and beets), and vegetables (cabbages, pumpkins, and carrots). Furthermore, the grazing areas would allow pigs to freely search for various herbs and wild fruits, as well as chestnuts and acorns. The nature of the feeds used in these traditional diets was a limiting factor for the establishment of larger herds. Consequently, the small number of individuals per farm and the rudimentary animal facilities, prevailed as the main weak points of this production system, limiting productivity while possibly harming animal's welfare (ANCSUB, 2022b; Santos Silva et al., 2019).

Nowadays the majority of BI farms operate on free-range systems, most of them allowing access to pasture (Paixão et al., 2018), and producers generally possess licensed housing for pigs, providing better animal conditions. These pig farms are characterized by their open areas where pigs live, except during parturition and lactation. During these periods, sows and piglets are found in appropriate maternities. Some breeders also use a free outdoor system (camping) during all the productive cycle, complemented with thermally isolated shelters and maternities. BI pig production can be divided in four groups namely reproductive sows, boars, piglets, and fattening pigs (ANCSUB, 2022b; Santos e Silva and Nunes, 2013; Santos Silva et al., 2019). The average number of pigs per herd has increased to 30, which are still commonly raised in small farms, in highlands or mountainous areas 400 meters above the sea level in the North of Portugal (Paixão et al., 2018). It is estimated that 70% of all born piglets are slaughtered between 8 and 12 kg, to produce one of the most typical BI products, roasted piglets. The remaining piglets are raised, fattened, and processed into traditional transformed products. Consequently, weaning is also early, occurring when piglets are 40-45 days old. BI pig production includes two growing periods. The first is characterized by a moderate-fast growth until 70-75 kg BW, followed by a fattening-finishing period until 120-180 kg BW, during which different diets are consumed, depending on

the local availability of agricultural products and by-products which can include flour, various fruits, vegetables, nuts, and acorns. The growth rate at this last period evidently varies within farms and ultimately relies on the feeding management and the opportunity to provide the animals with access to pasture in open areas (Santos e Silva and Nunes, 2013). The average BW of adult BI pigs can attain 180 kg in boars, and 150 kg in sows. Growing and finishing pigs may be reared in mixed systems in which the animals are bound to an open park area or enclosed in stables with access to grazing areas. The availability and extensiveness of pasture areas, together with the total number of individuals per herd, dictate whether the system can be described as intensive, semi-extensive, or extensive. The most common diet continues to include a base food, generally a mixture of cereals, which is supplemented with tubers, vegetables, and fruits along the year. An alternative approach to improve the traditional feeding strategy has been developed through the inclusion of cereal concentrates and some commercial feeds. However, resorting to commercial feeds only takes place at specific periods of the productive cycle such as lactation and weaning (ANCSUB, 2022b; Santos e Silva and Nunes, 2013; Santos Silva et al., 2019). In farms following traditional systems, BI pigs are fattened slowly and slaughtered within 1-2 years of age (with 120 to 180 kg BW), which is planned to occur during the coldest months, between November and February. Data on the intake and nutritional value of the feeds given is very limited and narrows the overall assessment of BI growth potential. Following semi *ad libitum* feeding conditions, average daily feed intake is approximately 1.7 kg/day during the growing period and can increase up to 2.7 kg/day during the fattening period. ADG during the growing period vary between 546 and 563 g/day, while in the fattening period ADG varies between 343 and 653 g/day. These BI growth potential results were obtained in several studies and farms and illustrate the great variability found within each rearing period and farm. The high heterogeneity of these values and the low to moderate growth differences can be associated to the nonuniform production system applied by each producer, including the intensification levels and feeding conditions (Araújo et al., 2016; Santos e Silva et al., 2000; Santos Silva et al., 2019).

Since long, BI sows have been praised for their fertility and prolificacy (Pinto, 1878). On the other hand, the maintenance of boars in small pig farms involves high economic costs which is why breeders have reduced boar numbers and some are starting to resort to artificial insemination (Paixão et al., 2019). However, to improve the currently results achieved by artificial insemination it is recommended to improve the conditions of semen transport and storage in the farms, while promoting farmers training in reproductive management. In small-scale farms with fewer breeding sows, mating occurs in community facilities and is performed by the “village boar”. This reproduction scheme holds clear disadvantages regarding contagious diseases, higher inbreeding risks and higher reproductive intervals, but is a cheaper method than artificial insemination (Santos Silva et al., 2019). Table 3 features a summary of various reproductive traits of BI pigs. First parturition for BI sows occurs between 10-12 months of age, averaging 1.9 litters per year with 9.3 piglets born per litter, each with a BW at birth around 1.8 kg. The weaning period is much variable, being performed between 28 and 60 days (7.2-11.1 kg BW), depending on the intensification of the system. The mortality rate, either at birth or at weaning, is also

variable between farms, although intensive systems generally present higher death rates. Farrowing intervals are higher in farms following more extensive/traditional production systems, due to management decisions such as the increased weaning period (up to 60 days) and the more extensive rearing and feeding conditions. However, piglets raised in extensive production systems are heavier and sold at higher prices (Santos Silva et al., 2019).

Table 3. Summary of the main reproductive traits of Bísaro pigs (Adapted from Santos Silva et al. (2019)).

Parameter	Production system		
	Intensive, Outdoor	Semi-Extensive, Outdoor	Traditional
Age at first parturition (months)	11.6-11.9	11.3	10
Litters per sow and per year	1.6-1.8	2.2	1.5
Piglets alive per litter	6.9-9.1	10.0	12.0
Live weight at birth	1.7-1.8	1.7	NA
Stillborn per litter (%)	9.8-11.0	5.0	8.3
Mortality at weaning (%)	20.5-34.8	14.3	16.6
Lactation period (days)	42-52	28	60
Farrowing interval (days)	200-203	162	232

NA = not available.

1.2. Meat quality, carcass traits, and consumer perception

1.2.1. Nutritional value of meat

Meat is one of the most important food resources to humans and is generally seen has the central part of a meal. Since the Middle Age, eating meat has been a symbol of wealth and fortune. Today's meat-eating patterns have widely expanded since then, and today, as the world and population wealthiness have developed, more people can afford to purchase meat. Meat and animal protein resources have become a regular presence in the modern eating habits, with increasing demand (Horowitz, 2006). In the last two decades, for example, we have witnessed a constant increase in the world's average supply of protein of animal origin, attaining a maximum of 31.6 g/day and per capita (Table 4).

Table 4. World's average supply of protein of animal origin between 2001 and 2017 (FAOSTAT).

Year	Animal protein supplies (g/day per capita)
2001	26.8
2002	27.0
2003	27.3
2004	27.6
2005	28.0
2006	28.6
2007	29.1
2008	29.5
2009	29.8
2010	30.0
2011	30.4
2012	30.7
2013	30.8
2014	30.9
2015	31.1
2016	31.3
2017	31.6

Meat is an important nutritional resource, mainly due to the high contents in protein (ranging from 12.3% up to 34.5%) and essential amino acids, iron, and various other minerals and vitamins (Ahmad et al., 2018). Proteins constitute the building blocks of life, providing support to

natural occurring processes in mammals, including growth, regeneration, hormone regulation, digestion, and oxygenation. Furthermore, diets rich in protein have the potential to induce weight loss because of the more filling effect of protein, besides the fact that protein requires more energy to be digested and presents an increased diet-induced thermogenesis when compared to other nutrients such as carbohydrates and fat (Pesta and Samuel, 2014).

Nevertheless, meat also contains moderate and variable amounts of fat, depending on the meat source and cutting. The fat tissue present in meat is composed of fat cells (adipocytes) that storage lipids, and it can be located between different muscles in a same cut (intermuscular fat) or within the same muscle (IMF). Lipids can be defined as triacylglycerols or esters composed of up to three fatty acids (FAs) and a glycerol molecule (Ahmad et al., 2018). Fat is generally known to be deleterious to the cardiovascular system and potentiate the risk of cancer development, so it is advisable to reduce the total quantity of fat consumed, particularly saturated fat. Consumers' attitude towards fatter meats has also evolved accordingly, with most people preferring meat with no visible fat (Kerry and Ledward, 2009; Wood et al., 2003). On the other hand, lipids, and particularly FAs, whether in the adipose or muscle tissues, can greatly influence the quality of meat or processed products and are central to the nutritional and sensory values of meat (Webb and O'Neill, 2008). Fat deposits can also play important roles in human and animal health. Primarily, fat is an energy storage unit, but it can also serve has a protective layer for organs (visceral fat) and be found under the skin (subcutaneous fat) where it also can contribute to regulate body temperature (Ahmad et al., 2018). Furthermore, the extensive autocrine, paracrine, and endocrine functions regulated by fat deposits in the body, through the release of several cytokines for example, have also been found to modulate gene expression at many metabolic levels, and drive nutrient flow (Trayhurn and Beattie, 2001). Besides the overall quantity of lipids found in meat, it is also important to consider the associated FA composition, which can depend on the feed of the animal. This is especially true in monogastric animals such as pigs and chicken (as in all vertebrates), which can efficiently reflect in their tissues the FA composition present on their diets, particularly regarding alpha-linolenic (C18:3, n-3), linoleic (C18:2, n-6) and long-chain polyunsaturated fatty acid (PUFA) contents (Ahmad et al., 2018; Kerry and Ledward, 2009; Wood et al., 1999). Including in the feed of pigs the appropriate PUFAs of plants and oil seed sources for example, enables them to be absorbed intact in the small intestine and directly incorporated into tissue lipids. Alpha-linolenic and linoleic acids can then serve as precursors for synthesizing arachidonic (C20:4, n-6) and eicosapentaenoic (C20:5, n-3) acids, respectively. Omega 3 and omega 6 FAs are mandatory in the diets of humans and other monogastric mammals since they cannot be synthesized by them. These are responsible for carrying fat-soluble vitamins such as A, D, E and K, and also for performing major regulatory roles in the immune response system (Webb and O'Neill, 2008). Dietary fat saturation greatly influences circulating cholesterol concentrations and the associated risk of developing cardiovascular diseases. Contrarily to saturated fatty acids (SFAs), unsaturated FAs are an important part of a healthier diet since these are responsible for lowering circulating cholesterol levels, particularly low-density lipoproteins (Feingold, 2000).

Foods of animal origin are also important sources of essential minerals including iron, potassium, phosphorus, sodium, magnesium, zinc, and selenium. Iron, in particular, participates in the respiratory process of oxygen and carbon dioxide transportation, as well as in the oxidative production of ATP in the mitochondria, and can occur in two forms, haem and non-haem iron. Heminic iron form is mainly present in haemoglobin and myoglobin, and therefore available in meat (including pork) and other animal sources. Presenting high degrees of bioavailability, it is efficiently absorbed, easily covering daily iron requirements in humans (Kerry and Ledward, 2009; Simpson and McKie, 2009).

1.2.2. Sensory meat quality traits

The study of carcass characteristics and meat quality has been challenging the scientific community for decades since quality is a complex concept. Qualitative points of interest shift in importance depending on which part of the meat industry chain is the product targeted to. For example, for the fresh meat market, appearance related traits such as colour and amount of visible fat are crucial to influence the decision of purchase from consumers, while for processed meat products, technological traits such as pH, water retention and oxidative stability are more relevant (Torres et al., 2013). Besides that, several elements influencing fat deposition, distribution, and composition on livestock carcasses remain unclear despite these processes being extensively investigated.

Wood et al. (1999) previously defined meat quality as the attractiveness of meat to consumers. The modern meat consumer takes into consideration the nutritional value of what he eats, as well as the taste and the associated sensory qualities that provide pleasure when he is eating (Torres et al., 2013). The three main attributes providing this value are tenderness, juiciness, and flavour.

Meat tenderness is basically determined by the muscular fibres (including IMF), the surrounding connective tissue, and its associated collagen and elastin contents. It is a key factor to determine global quality in fresh pork products, that strongly influences consumer satisfaction at consumption. Still, it is a hard parameter to efficiently estimate, together with the range of consumer's acceptability level and all influencing components at play (Purchas, 2014). Overall, tenderness of meat tends to be influenced by changes in the myofibrillar protein structure of the muscle, between the time of slaughter and the time of consumption. One factor affecting this is *post-mortem* refrigeration. When a carcass is refrigerated too quickly after slaughter, the muscle fibres contract and, consequently, the force required to shear them increases, particularly after cooking. Ageing and long conditioning times are two factors that contribute to meat tenderness through the action of proteolytic enzymes that degrade minor muscle proteins, fragmenting and weakening the muscle structure (Wood et al., 1999). Another factor that positively influences meat tenderness is the intramuscular fat content, while increased collagen content usually

lowers it. Finally, ultimate pH has a preferred range for better tenderness (6.2-7.0) even though at the expense of lowering other quality traits (Purchas, 2014).

Juiciness is characterized by the amount of water retained in a meat product. Lipid content can also positively influence juiciness, as it helps to trap moisture in the muscle, besides the fact that during the mastication process, the melting fat helps to preserve the juicy sensation (Torres et al., 2013; Wood et al., 2003). Meat ageing can also increase water retention, and therefore juiciness. On the other hand, evaporation and drip losses decrease juiciness. Other factors influencing juiciness include all those that directly or indirectly allow for changes in water retention in the muscle fibres and those that influence IMF content. Additionally, juicier meats boost the flavour component and stimulate saliva production (FAO, 2014; Torres et al., 2013).

The flavour component is many times associated with the aroma, and greatly contribute to a better eating quality. These perceptions can be affected by the animal species, feeding diet, meat cut, and the cooking and preservation methods employed (e.g. smoked or cured meat) (FAO, 2014; Torres et al., 2013). The chemical reactions provoked by these last processes lead to the breakdown of proteins and carbohydrates present in meat and it is what provides the particular flavour of cooked meat, with inosine, phosphate and ribose being great flavour precursors (Wood et al., 1999). Lipid degradation can also affect flavour through their resulting products. Increased flavour intensity is also to be expected with ageing or conditioning (Wood et al., 1999), although some authors refer the production of bitter flavours with the latter one (Rousset-Akrim et al., 1997).

1.2.3. Carcass and meat quality traits of the Alentejano and Bísaro breeds

Carcass composition traits are a decisive component in meat production industries since these should determine the market direction to take regarding sales, while positively influencing consumer interest towards the meat product. An efficient strategy allowing for the maximum usage of the produced resources, while lowering wastes due to excessive fat and bone content, for example, is of utmost importance. Thus, knowledge of carcass composition is essential for meat producers to target a specific market segment and increase profits, besides planning growth and manage animal nutrition efficiently. Generally, carcass composition traits can be described as the absolute or relative quantities of muscle, fat, and bone, including the total amount of protein and lipid contents, ash and water (Kerry and Ledward, 2009).

As mentioned before, meat quality related traits can vary with genotype and feeding conditions, among other factors, and pork is no exception. However, both AL and BI breeds present high heterogeneity regarding growth and carcass characteristics between and within herds. Moreover, few studies were performed under controlled experimental conditions to assess the genetic potential of each breed (Santos e Silva and Nunes, 2013).

Published studies report slower growth rates of both breeds when compared to modern ones (Neves et al., 2003; Pires da Costa et al., 2002), which can attain over 1000 g/day in optimal intensive conditions (Čandek-Potokar et al., 2019). The slower growth rates also relate with their higher feed conversion rates and low yield of primal lean cuts (Neves et al., 2003; Pires da Costa et al., 2002).

There are very few works comparing AL and BI genotypes in the same experimental conditions. In the work of Santos e Silva et al. (2000) AL presented higher carcass yield than BI pigs (78.9 vs. 77.0%). However, they also had significantly lower proportions of head (7.5 vs. 8.5%), shoulder (18.9 vs. 20.5%) and ham (25.4 vs. 27.0%), contrasting with the higher fat cuts such as belly proportion (22.5 vs 19.1%) which is suggested to compensate when estimating carcass yield. AL pigs also presented shorter carcasses (90.2 vs. 106.9 cm) and over 5 cm of backfat thickness, 170% more when compared to BI pigs (Santos e Silva et al., 2000).

These results agree with AL's medium sized bodies with lower potential for lean meat deposition and also acknowledge the higher propensity to accumulate body fat, a comparable adipogenic pattern to what occurs in the genetically similar IB pig (Muñoz et al., 2018). Intensively selected pig breeds present carcasses with much higher lean meat content and much lower fatness when compared to these two local genotypes (Dalla Bona et al., 2016; Lebret et al., 2014). On the other hand, BI's carcasses present intermediate yield performance for meat cuts when compared with AL and modern genotypes, while also displaying intermediate subcutaneous fat levels (Santos e Silva et al., 2000).

Regarding the chemical composition of the subcutaneous fat of AL pigs, previous studies have shown fat contents over 94%, with water contents around 5% and protein under 1% (Neves et al., 2004). This contrasts with BI's lower fat percentage which is usually found between 87 and 89%, and higher water content with values between 8 and 10% (Rodrigues, 2019). In relation to colour parameters, both breeds presented similar lightness (L^*, around 79), but different redness and yellowness, with AL pigs presenting higher redness (a^*, 3.7 in AL and 1.7 in BI) but lower yellowness (b^*, 4.2 in AL and 8.9 in BI) when compared to BI pigs. AL pigs also presented lower chroma (C^*, 5.6 in AL and 9.1 in BI) and hue angle (H^o, 49.6 in AL and 79.7 in BI) than BI due to a greater absolute difference in yellowness than redness (Neves et al., 2004; Rodrigues, 2019). Finally, the fatty acid composition is different between breeds with AL presenting overall less SFAs and PUFAs but more MUFAs. In AL, MUFAs can represent over 59% of the total FAs, while SFAs attain on average 26% and PUFAs 15%, with the most represented FA, oleic acid, reaching over 53% (Neves et al., 2007). In BI pigs MUFAs can represent over 44% of the total FAs, SFAs 37% and PUFAs 18%, with oleic acid reaching only about 38% (Carvalho, 2009).

The *Longissimus thoracis et lumborum* muscle of intensive raised AL pigs have previously showed significantly lower protein values but higher total fat, pigments and dry matter when compared to commercial Landrace x Large White pigs raised under the same conditions (Teixeira and Rodrigues, 2013). In another study, pH measured at 45 min *post mortem* and ultimate pH measured at 24h *post mortem* in the LL muscle were higher in BI when compared to the AL

genotype. However, when comparing to a modern Landrace x Large White cross which can be as low as 5.35, both Portuguese breeds presented higher pH values (Santos e Silva et al., 2000). This modern type also showed worse values for lightness (*L**, 56.98), drip loss (3.63%) and cooking loss (20.61 %) which indicate better sensorial quality and technological characteristics in the AL and BI breeds' meat. The LL muscle of AL was also estimated to be 22% more tender than BI pigs using the Warner-Bratzler Shear Force (WBSF) test. Protein content was also significantly lower in AL when compared with BI (21.82 and 22.98%), with the highest values for the modern type (23.87%). In contrast, IMF content in AL attained approximately 4% of the muscle which was 76% higher when compared to BI and 241% higher when compared to the Landrace x Large White type (Santos e Silva et al., 2000). The FA profile of the IMF portion is more similar in the *Longissimus* muscle of both breeds, although AL pigs tend to display higher MUFAs and lower PUFAs content. Overall, in AL MUFAs represent 50% of the total FAs, SFAs represent 42% and PUFAs only 7%, with the most represented FA, oleic acid, attaining over 45% (Teixeira and Rodrigues, 2013). In BI pigs, the FA profile is similar to the one observed in the adipose tissue, with MUFAs representing 43% of the total FAs, SFAs reaching 38% and PUFAs 19%, with oleic acid only attaining for 38% (Carvalho, 2009).

1.3. Molecular genetics to improve pig breeding programs

1.3.1. Evolution of breeding programs

Selective animal breeding comprehends the improvement of specific phenotypical traits of interest within a determined species or breed. The individuals showing the best potential are selected as parents to genetically improve the traits of interest (Hill, 2001). Animal breeding is, of course, based on the fact that offspring reflect almost entirely the traits of their parents (heritable traits) and the Mendelian principle that in diploid individuals 50% of the DNA, including the heritable ability for that specific trait, is carried to the new generation. Usually, the phenotypical characteristics are measured in all candidate individuals or related ones, considering the genetic data available for that population. This data is then used in proper statistical models to estimate the genomic breeding values. These values are the selective component that grade the potential of individuals to a specific trait (Oldenbroek and Waaij, 2014).

Animal genetic improvement goes hand in hand with human history and started as early as animal domestication. However, animal breeding did not occur selectively until the late 1700's. People crossed their animals with no intention of maintaining or introducing specific characteristics until Robert Bakewell first started to register accurate animal performance and reproductive data in the United Kingdom. Bakewell was encouraged with the idea of breeding "the best to the best", so he mated related animals with the same traits in order to fix them. Furthermore, in 1791 the first herdbook was created in England and included the pedigree and family history of race-winning horse breeds (Wykes, 2004). Knowledge has evolved since those times, particularly during the 19th century with the scientifical breakthroughs by Darwin and Mendel. The first identified the natural selection pressure occurring within any specific environment, with the best adapted individuals having the highest chance for survival and reproducing. Mendel, on the other hand, studied the basics of genetic inheritance using pea plants and demonstrated that gene alleles can have a dominant, recessive, or additive profile. These findings were followed by the development of the theorem of population genetics, presented in 1908, which later became known as the Hardy-Weinberg equilibrium. They stated that within large populations where random crossing is occurring, and in the absence of selection, migration, mutation and random drift factors, the genotype and allele frequencies remain constant and do not change across new generations (Martins et al., 2019). These advances and others have propelled the creation of the modern animal improvement theory, during the first half of the 20th century. In 1937, the father of modern animal improvement, the American Jay Lush, published the book "Animal breeding plans" which would influence animal breeding at a global level. His genetic breeding programs, based on the previous studies of Ronald Fisher and Sewall Wright, would rely on the genetic value of the animals, characterized by a combination of quantitative statistics and genetic data, instead of purely subjective visual traits (Martins et al.,

2019; Oldenbroek and Waaij, 2014). In 1953, Watson and Crick discovered the double helix structure of DNA, which later allowed genomic selection in breeding programs. Genomic selection brought new value for breeding programs since the producers no longer had to wait for the animals to grow into fully developed adults to check their resulting phenotypical traits. The association between DNA composition and animal performance enabled a much faster and efficient method to improve breeding programs. Furthermore, genomic selection can also be much useful for tracing disease related traits, in order to prevent them to develop, in as many animals as possible (Goddard and Hayes, 2007; Oldenbroek and Waaij, 2014). More recently, Meuwissen, Goddard, and colleagues, provided a way to incorporate complete genomic sequences into animal models, such as the one previously developed by the statistician Charles Roy Henderson, taking advantage of today's increased computer power to better estimate genomic breeding values. Nevertheless, incorporating full genomic sequences into estimating breeding values is still too expensive and is not yet common practice, particularly in small local farms (Meuwissen et al., 2001; Oldenbroek and Waaij, 2014). On the other hand, the animal model developed by Henderson (Henderson, 1984) and known as BLUP (Best Linear Unbiased Prediction) is still one of the most used methods to estimate breeding values. BLUP has the advantage to include a matrix with data from all relatives and also allows for the correction of phenotypes for systematic influences. However, these corrections for environmental factors require sufficient genetic links between environments in order to better estimate the effects of those conditions. In other words, enough individuals of similar genotype should be present across the different environmental influences (Oldenbroek and Waaij, 2014).

In Portugal, animal conservation and improvement programs were firstly introduced in the 1990s for most of the local breeds of cattle, sheep, pigs, and goats. These were followed by the creation of the herdbook for each breed. Nowadays, each breeders association has a specific conservation and improvement program, according to the goals specifically set by the breeders (Martins et al., 2019). These goals, however, usually tend to study and characterize the population while addressing issues like the low number of individuals and the implied diversity and inbreeding issues, and not improve specific phenotypical traits (ANCPA, 2022; ANCSUB, 2022a).

Several AL traits that would benefit from improvement programs of the breed, without jeopardizing its ability to adapt to the local ecosystem as well as the product's quality, are identified. Firstly, it is important to increase AL's prolificacy, precocity in having litters, and its maternal competence. Additionally, it would also prove valuable to increase the number of functional teats per sow, as well as the feed conversion ratio and the muscle to fat ratio. Nevertheless, the low number of AL individuals, and the inbreeding issues associated with that, have so far challenged the application of improvement programs. AL pig producers have previously attempted to improve the breed by crossing AL genotypes with leaner commercial breeds like Landrace, Tamworth and Yorkshire, particularly during the second half of the 20th century. However, these attempts failed to generate the genetic improvement desired and risked lowering the genetic variability of the AL breed (Gama et al., 2017a; Marques, 2001). In Spain,

however, the cross between IB and Duroc breeds has successfully improved the eating quality of the meat of the resulting pigs comparing to the Duroc and the respective productive parameters comparing with the IB, in practices that have been implemented for over 40 years (Nieto et al., 2019; Ramírez and Cava, 2007a, b).

Today's ongoing genetic conservation and improvement program for the AL breed, approved under the guidelines of the *Portaria* 268/2015 of September 1st, is managed by ACEPA, A.C.E. (Alentejano Pig Complementary Consortium of Companies, A.C.E.), created in 2011, of which ACPA and ANCPA are part of (Charneca et al., 2019). Choosing a vast panel of molecular markers that can be useful in selection programs require much data about the link between phenotypical traits and genetic markers. Due to time and budget constraints, the association has focused its interest in previously suggested genetic markers, rather than massive surveying for these (Gama et al., 2017c). The completed activities within the scope of the program include 2017 surveys for the Halothane gene prevalence (3% of estimated allele frequency, with no homozygous nn detected in 140 AL pigs, Gama et al. (2017b)) and for the favourable G allele (94% of estimated allele frequency in 140 AL pigs) in the genetic marker rs81273273, associated with higher concentrations of vaccenic acid (Gama et al., 2017c). It was also performed a genetic characterization through a demographic analysis (performed every three years) of the registered reproducing population born between 2003 and 2019 (Gama, 2020). Only 5% of all registered AL pigs had both parents registered which resulted in only 30 090 animals considered of 289 farms, with the rate of ancestry knowledge drastically declining particularly since 2011. Furthermore, approximately 8% of all matings were consanguineal (0.44% per year) and 53% of all pigs born after 2017 were inbred (Gama, 2020). Other planned actions of this association include carcass and meat quality studies (every three years), collection and maintenance of genetic material in the *Banco Português de Germoplasma Animal*, and artificial insemination, although the later to a less extent (ANCPA, 2022).

For the BI breed, ANCSUB is the responsible association for the preservation and management of this genetic resource, and its performed activities also follow the guidelines determined by the same legal act, submitted by the Portuguese Ministry of Agriculture and Sea. These activities include demographic (Martins et al., 2018) and prolificity (Martins and Silvestre, 2020) evaluation studies of the BI population. Contrarily to the AL population, most BI pigs registered had both parents known and registered (200 336 out of 204 531, 97.5%) and were found throughout 425 farms. However, over 54% of all animals are inbred, and these presented an average of 19.42% of consanguinity which revealed severe inbreeding concerns (Martins et al., 2018). Regarding the prolificity data, BI pigs present an average of 9 (±3 SE) piglets per litter, when considering all prolificity records obtained from 1995 until 2020 (approximately 40 000). BI pigs are indeed more prolific than AL pigs, but still present low results when compared to commercial breeds raised in intensive production systems. Furthermore, the genetic value of the prolificity trait in the BI pig population reached its peak in 2016, and has been consistently decreasing since that time (Martins and Silvestre, 2020).

1.3.2. The role of molecular genetics in the present and future of breeding programs

Molecular genetics involves the study of the link between genotype variation and a corresponding phenotype, which is especially useful for improving animal breeding programs. Traditional breeding systems are more challenging and expensive since they rely on the collection of phenotypical data, taking a longer time to properly estimate the individual breeding values. As previously mentioned, genomic selection has proved to be a very powerful scientific development leading to faster and more efficient breeding programs. Nowadays, more animal breeders' resort to genetic data to breed more productive food animals, particularly in commercial breeds, minimizing the required environmental resources in the process (Tan et al., 2017; Van Eenennaam and Young, 2019).

As genetic selection aims to maximize the rate of increase of the genetic merit of a given target trait, some traits tend to present a nonlinear pattern. These traits are usually known as complex traits. Complex quantitative traits are, by definition, phenotypical traits influenced and regulated by numerous genes, and they possibly interact with environmental factors. In a population with enough genetic variability for a given function where a genetic basis can be directly associated to it, it is possible to improve the genetic value for a trait by selecting the best individuals as parents (Gianola and Rosa, 2015). However, these complex traits are not easily explained by the Mendelian inheritance principles of single genetic factors as their inheritance remains unresolved. Nevertheless, the development of complete genotyping assays has allowed genotype determination of thousands of individuals, discovering several single nucleotide polymorphisms (SNPs) that have revolutionized the study of complex traits (Goddard et al., 2016).

Most of animal traits are determined by a set of various genes and are therefore recognized as complex, such as body weight and milk production (Oldenbroek and Waaij, 2014). In the past, assessing the genetic basis and individual merit for a determined trait in an animal or breed would be performed based only on the analysis of observed phenotypical traits and genealogical information. This data can nowadays be complemented with the extensive amount of molecular markers that genomics allows us to identify and associate with any given trait (Gianola and Rosa, 2015).

Genomics is essentially the study of the structure, function, and evolution of genomes. Originally, molecular genetics were used to study single candidate genes that were known to code for a protein directly involved in the development or function of a trait, searching for mutations in that candidate gene that could alter the phenotypical result. Polymorphisms found would then be statistically associated or not with that trait. However, multicellular organisms, especially mammals, often present large genome sequences and soon the arrival of genetic mapping would revolutionize the search for genetic markers. When a small portion of DNA is found to substantially influence a particular trait, it is acknowledged as a Quantitative Trait Locus (QTL). One of the first studies using the method of QTL mapping was performed in a wild boar x

Large White crossed pig by Andersson et al. (1994), in which a region of chromosome 4 was accounted for the phenotypical variation found in several traits including growth rate, fatness and length of small intestine. Both strategies have added value and can be combined to identify more accurate candidate genes for given traits. Firstly, QTL detection would survey the main genome regions that affect a trait and then a candidate gene search can be carried out within those QTL regions with extra assurance of good results. Other QTLs have been previously described to affect traits in pigs, being as diverse as oleic acid content, intramuscular fat, teat number and coping behaviour. As of June 6 2022, a total of 35384 QTL associations have been detected in the pig genome and submitted to the public database PigQTLdb, covering 762 publications, representing 716 distinct phenotypical pig traits, spawned throughout 288 genes (Animal_QTLdb, 2022). Table 5 summarizes the top 20 traits with more QTL associations found in pigs.

Table 5. Summary of the top 20 traits with more QTL associations found in the genome of pigs.

Trait	Number of different QTL associated
Drip loss	1092
CD8-negative leukocyte percentage	900
Intramuscular fat content	891
Palmitoleic acid content	864
Stearic acid content	848
Saturated fatty acid content	831
Average daily gain	815
Coping behaviour	813
Palmitic acid content	803
Oleic acid content	793
CD8-positive leukocyte percentage	778
Monounsaturated fatty acid content	750
CD3-negative, CD8-negative leukocyte percentage	713
Teat number	664
Average backfat thickness	586
Mean corpuscular volume	558
Loin muscle area	415
Feed conversion ratio	410
Sperm motility	397
Mean corpuscular haemoglobin concentration	388

In September 2003, the Swine Genome Sequencing Consortium was established, and with it, the assumed goal of obtaining the first complete genome sequence of a pig, in this case a female Duroc. This was successfully achieved and published in 2012, using shotgun Sanger Sequencing and bacterial clones, later complemented with Illumina's next generation sequencing

(NGS) data (Groenen et al., 2012). Since then, genomes of hundreds of pigs of different breeds have been sequenced to study the effects of genome variation, evolution and selection throughout history (Groenen, 2016).

Similar to QTL studies, genome wide association studies (GWAS) make use of genome sequences and associate the existing genetic variations to specific traits, so that phenotypes can be predicted. The first GWAS attempt occurred almost two decades ago. Although the coverage of the human genome in that study was low (only 71%) and only 100 000 SNPs were used from the Japanese Single Nucleotide Polymorphisms database (Ikegawa, 2012), this study represented the first step towards the easier, more affordable and widespread versions we hold today. Instead of singular genes or small fractions of DNA like in QTL studies, GWAS survey genetic variation on a genome-wide scale. Massive SNP genotyping of pigs through chips has also become a reality with three options available in the market at the moment from Illumina (over 64k SNPs, (Ramos et al., 2009)), Neogen (over 70k SNPs, (Muñoz et al., 2019)) and Affymetrix (over 658k SNPs, (Thissen et al., 2019)).

Major improvements in genetic traits can either involve higher selection intensities, more accurate predictions of the genetic merit, or be obtained through reducing the generation intervals. However, considerable increases in the selection intensity factor have to be taken cautiously since they can result in losses regarding genetic diversity. On the other hand, collection of whole-genome data can also be useful to trace and properly manage potential diversity and inbreeding issues in the populations (Cervantes and Meuwissen, 2011). For example, in the case of pig populations, which already present short generational intervals, increasing the prediction accuracy is one of the main focus points for improving genetic traits, particularly in local breeds with limited number of individuals, and implicit diversity and inbreeding problems (Stock and Reents, 2013).

On the other hand, full knowledge of genomes and recent developments of sequencing techniques have enabled the expansion of functional/expressional genomics. In multicellular organisms, the DNA present in the nucleus of every cell contains identical genomic data, except for the gametes and neoplastic cells. The transcriptome, on the other hand, fluctuates widely according to the cell type, their respective function, and their current state. The transcriptome encompasses all RNA transcription molecules (including messenger, ribosomal, transfer and micro RNAs) present in a determined cell or tissue and its profile dictates the expression of all genes, regulating the multitude of metabolic processes occurring. Messenger RNA (mRNA) is the intermediary element that transports the genetic information from the nucleus to the cytoplasm, where protein synthesis will occur. When genes are being expressed, several copies of their corresponding mRNA particles, produced via transcription, are present so that by assessing the various existing mRNA at a given moment, the status of the various metabolic processes occurring within the cells/tissues can be evaluated. For some time, microarray analyses remained the most commonly used method to characterize gene expression, granting a high throughput analysis of numerous genes simultaneously (Frese et al., 2013). Because mRNAs are easily

degraded, these are converted to the more stable complementary DNA (cDNA) form. Microarrays make use of microchips with anchored probes (short DNA sequences) that hybridize to known target genes, since complementary sequences bind to each other, allowing the study of their expression. Microarray technology appeared to replace techniques such as Northern Blot and reverse transcription polymerase chain reaction (rt-PCR) which only allow the testing of a few selected target genes, while microarrays unlock a more global expression profiling (Govindarajan et al., 2012).

Nowadays, an increased interest on alternative RNA sequencing protocols have seen the rise of a technology often referred to as RNA-seq. This method is one of the main applications of NGS technologies and allows the study of the occurring complex cellular processes through the identification of DEGs within distinct experimental groups. RNA-seq has the advantage of not requiring previous knowledge of the exact location of the targeted genes, while providing an affordable, fast, and unbiased sequencing method. Furthermore, a reference genome (although recommended) and specific predesigned probes are not mandatory. When compared to microarrays, RNA-seq experiments also require fewer amounts of RNA, have less associated noise issues, and provide direct knowledge of splicing events and new isoforms (Frese et al., 2013).

The workflow of RNA-seq can slightly differ between the options available in the market, although it comprises several shared steps (Figure 3). After sampling, total RNA is extracted from the target tissue or cell culture, and then fragmented into small pieces. Then, reverse transcription of the resulting fragments produces the cDNA that will be used to assemble the libraries together with the help of specific adapter sequences.

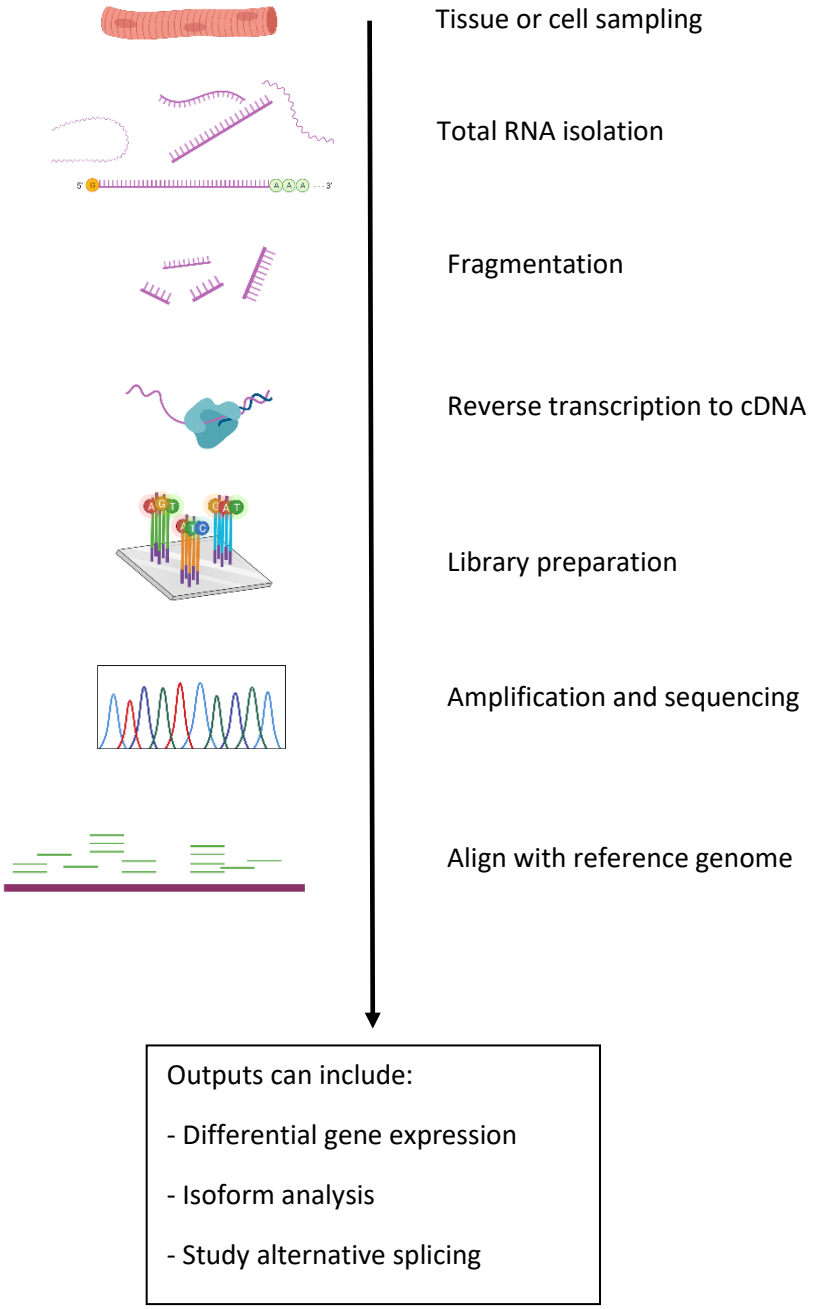

Figure 3. Overview steps of an RNA-seq experiment (Kukurba and Montgomery, 2015).

Library construction protocols depend on the RNA species to be detected, such as ribosomal RNA (rRNA), precursor mRNA, mRNA, and noncoding RNA. During these steps, certain properties can be changed to enhance the detection of determined target RNAs and limit the

presence of others. This is particularly useful since rRNA can account for up to 95% of the total RNA present in most cell types. When for example mRNAs are the target of an experiment, rRNA transcripts should be removed before library construction or they will deplete most of the sequencing reads, limiting target detection, and reducing the overall depth of sequencing coverage. This is why numerous protocols aim at selecting for polyadenylated RNAs using poly-T oligos covalently attached to a substrate, limiting the presence of rRNAs while supporting a successful transcriptome profiling (Kukurba and Montgomery, 2015).

Illumina sequencing platforms remain the most used in the sequencing industry, estimated to be around 80% of the global market (Mobley, 2021). These rely on fluorescently labelled reversible-terminator nucleotides that are clonally amplified while restrained to the surface of a glass flow cell. Sequenced reads are then assembled into a reference genome or *de novo* using contiguous transcript sequences if no reference genome is available (Bentley et al., 2008; Kukurba and Montgomery, 2015). Generally, in expression studies, sequenced reads have between 50 to 250 bp, since higher read lengths present lower sequencing accuracy and tend to be more expensive (Tan et al., 2019). The number of reads identified for each determined genome location allows a proper estimation of the expression levels for each gene.

One of the main outputs of RNA-seq experiments, differential expression analysis, aims to identify the genes that were transcribed into significantly different amounts of mRNA between two or more biological groups. Nevertheless, since the true mRNA quantities are not directly measured, statistical tests are required to discern whether a gene is being overexpressed or not based on the amount of reads obtained (Li and Li, 2018). Normalization of reads is required to remove technical biases in the data, such as depth of sequencing, library fragment size and sequence composition bias, and enable gene expression comparisons within each sample and across all samples. The most common software tools to estimate differential expression include CuffDiff, BaySeq, DEGSeq, EdgeR and DESeq. DESeq2 is an R package for differential analysis of count data, using shrinkage estimation for dispersions and fold changes to improve stability and interpretability of results, allowing it to be a good solution for a large range of data types and applicable in studies with either low or high replicates. DESeq2, following the DESeq method, firstly proposed by Anders and Huber (2010), assumes that most of the genes are not being differently expressed and uses the median of the ratios of observed counts to estimate size factors and normalize the data for each sample (Bedre, 2021; Love et al., 2014).

Following DEGs identification, several functional enrichment analyses using bioinformatic tools can be used to trace which metabolic pathways and gene networks are most associated with such DEGs. These can include programs like the Ingenuity Pathways Analysis (IPA) software (QIAGEN, Redwood City, CA, USA) (Krämer et al., 2014), the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009), and PathVisio (Kutmon et al., 2015), among others.

Most of RNA-seq studies still involve proper technical validation through another independent method, being RT-qPCR the method of choice. Good correlation results between

qPCR and various RNA-seq pipelines have been previously reported (Everaert et al., 2017). Only a small fraction (approximately 1.8%) of these genes were found to be extremely discordant regarding the direction of expression and significance levels, usually happening in shorter and/or low expression genes. On the other hand, and depending on the workflow, 80 to 85% of the total genes present concordant results between both methods. However, the requirement of a validation method is not unanimous within the scientific community and many authors defend that, unlike microarrays, RNA-seq experiments are more reliable and do not present such issues regarding reproducibility and bias when performed with sufficient biological replicates. When the expression of the given targets is low or the experiment is low on the number of replicates, it is recommended to validate it with an alternative approach such as RT-qPCR (Coenye, 2021).

Meat scientists aim to identify new meat quality biomarkers that can be assessed on living animals or on the carcasses at early *post-mortem*. The first option can be improved by using molecular genetics as a surveying tool, allowing the application of better genetic selection programs and predict the outcome of traits of interest, influencing breeding strategies to achieve the prospected quality levels. The latter can guide meat production systems towards better decision making, improving carcass distribution and specific cuts to the appropriate markets (Picard et al., 2015).

The identification and study of expression biomarkers has shed insight about the interactions between genetic and environmental parameters that influence the development of complex traits. In pigs, numerous examples can be found. Hamill and colleagues, for example, associated lower shear force values (higher tenderness) in the LL of pork with a decreased expression of protein synthesis related genes and an increased expression of protein catabolism related genes (Hamill et al., 2012). RNA-seq studies also have the potential to uncover genetic variants responsible for several phenotypical differences. One of the first porcine studies to make use of high throughput sequencing techniques was published in 2009 using 454-sequencing, and compared the transcriptome profile of cardiac muscle and skeletal muscle tissue (LL) (Hornshøj et al., 2009). This was followed by a stream of other studies in the past decade. For example Puig-Oliveras et al. (2014) managed to explore the LL muscle transcriptome of IB x Landrace crossed sows presenting extreme FA composition profiles. A total of 131 DEGs were identified, mostly associated with the lipid metabolism pathway, 18 of which were located in a previously determined genomic region of interest influencing IMF composition in pigs. In 2018, Horodyska and colleagues selected pigs diverging in feed efficiency and performed RNA-seq analysis to identify the molecular mechanisms influencing this complex trait with considerable environmental and economic value (Horodyska et al., 2018). Other studies have also compared the genotype effects on the transcriptome, particularly within European pig breeds, and attempted to associate it with the observed phenotypical variance (e.g. Ayuso et al., 2015; Benítez et al., 2019; Li et al., 2016a; Núñez et al., 2021; Piórkowska et al., 2018).

As previously mentioned, most European local pig breeds have been scarcely selected throughout the ages, either for better productive or reproductive traits. Portuguese local AL and

BI breeds are good examples of this, and any minor improvements that have happened are due to empirical selection performed by farmers. The application of genomic selection tools and other approaches in farms should be taken cautiously since AL and BI breeds still do not present a strong, homogeneous and large base populations. Furthermore, the lack of genealogical records in some farms and the somewhat still expensive (although reducing) cost associated with these methods also limit their application. On the other hand, selection based on genetic markers should be performed with prudence since these breeds hold an historical genetic uniqueness and an antagonistic genetic relation between productive/reproductive traits and quality traits has been previously demonstrated (Fernández et al., 2003). As a way to overcome these issues, and as it has been suggested for the Alentejano genetically similar IB breed, selection strategies should focus on boosting the yield of premium cuts without jeopardizing meat quality standards (Juan, 2014). In that way, we could profit from the use of genomic selection tools, recognized for improving traits of commercial pigs up to 50% (Knol et al., 2016).

1.4. Physiology and Metabolism overview

1.4.1. Adipose tissue

Fat is one of the main parameters determining good carcass and meat quality traits in farm animals. Adipose tissue in the body can occur between organs (visceral), under the skin (subcutaneous) in several layers separated by connective tissue, between muscles (intermuscular) and within the muscle (intramuscular). Adipose tissue was firstly described for its energy reservoir functions, but nowadays adipocytes (basic fat cells) are no longer seen as only triacylglycerol energy stores, and their extensive roles influencing autocrine, paracrine, and endocrine functions, by secreting hormones, adipocytokines and regulatory proteins, have been established (Poklukar et al., 2020; Richard et al., 2000; Trayhurn and Beattie, 2001). Furthermore, adipose tissue can have a shielding effect and provide thermic isolation, regulating heat loss (Gregory, 1989). Although various cell types are included within the adipose tissue matrix, adipocyte cells are present in higher numbers and determine the adipose tissue type (Richard et al., 2000).

Historically, adipose tissue has been classified for holding two visually distinct adipocyte types, white and brown. White adipocytes usually present a rounded shape, containing a single but large lipid droplet that can occupy most of the cytoplasm, pressing the rest of the organelles into the cell's periphery. Brown adipocytes, on the other hand, are characterized for holding numerous lipid droplets and iron enriched mitochondria, the latter being responsible for the brownish tone of these cells and of the brown adipose tissue (Figure 4). White adipose tissue is the most abundant type present in mammals and its main purpose is to store energy, besides providing endocrine communication and controlling insulin sensitivity levels. On the other hand, brown adipocytes can regulate body temperature through lipid oxidation mediated thermogenesis, elevating the basal metabolic rate, and are particularly present in hibernating animals (Richard et al., 2000; Smorlesi et al., 2012).

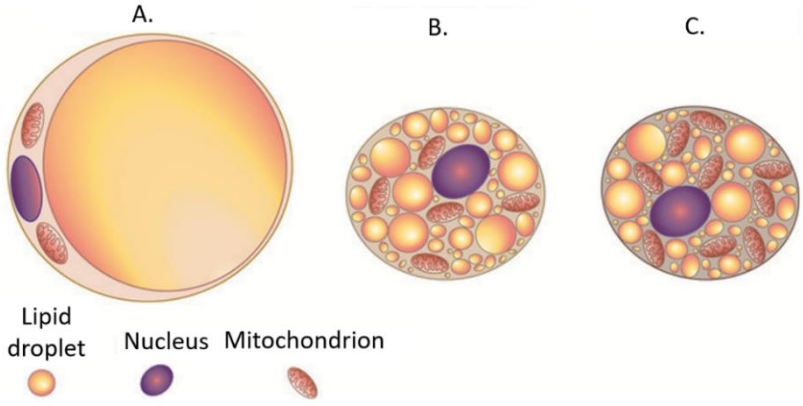

Figure 4. Different types of adipocytes: A. White adipocyte; B. Beige adipocyte; C. Brown adipocyte (Adapted from Paul (2018)).

More recently, two other adipocyte types have been described, namely beige and pink. Pink adipocytes are suggested to be formed via white adipocyte differentiation and present epithelial-like features responsible for developing milk-producing alveoli, granting its singular pink tone (Giordano et al., 2014). On the other hand, beige adipocytes (or "brite", as brown-in-white) stand between white and brown adipocytes regarding most characteristics. Usually, beige adipocytes develop subcutaneously through *de novo* differentiation of preadipocytes or through conversion of pre-existing mature unilocular white adipocytes when exposed to certain stimuli such as cold or β3-adrenergic agonists (Wang and Seale, 2016). However, maintaining the beige phenotype requires continuous stimulation since the adipose tissue is flexible and interchangeable. Like brown adipocytes, beige adipocytes have the ability to use stored energy and convert it into heat (Richard et al., 2000). The thermogenic effect of both brown and beige adipocytes is found associated with the expression of the uncoupling protein 1 (UCP1), responsible for catalysing the entering flow of protons (H^+) from the intermembrane space into the inner mitochondrial matrix. This leads to an increased respiratory chain activity that increases the amounts of available energy converted to heat. UCP1 activity is naturally inhibited by purine nucleotides. However, certain stimulating factors such as catecholamines, secreted by nerve cells as a response to low temperatures, leptin and other compounds, can trigger the signalling cascade in brown and beige adipocytes, inducing lipolysis and UCP1 activation upon binding of the released FAs (Wang and Seale, 2016).

Study of the processes responsible for inducing the "beiging" and "browning" of white adipose tissue have the potential to prevent obesity, insulin resistance, type 2 diabetes and other associated metabolic dysfunctions. Constituents of the adipose tissue, aside from the adipocytes, such as neurons, blood-vessel associated cells and immune related cells also play a defining role in the processes of differentiation and function of brown and beige adipose tissue. Cells of the type II inflammatory cytokine signalling pathway have been suggested to assist the "browning" and "beiging" of adipose tissue. Increased levels of interleukin (IL)-4 and IL13, for example, contribute for an alternative activation of macrophages which stimulates thermogenesis and the "browning" remodelling of adipose tissue through the secretion of catecholamines (Wang and Seale, 2016).

The subcutaneous fat depot in pigs is divided in two or three well individualized layers, separated by connective tissue, with the most inner layer usually presenting a higher proportion of SFAs and the external layer a higher proportion of desaturated FAs. In AL pigs, a fourth layer of fat can be developed in pigs raised over 120 kg of body weight (Neves, 1998). The rate at which lipid deposition naturally occurs in the various deposits of the body is influenced by genetic predisposition, animal growth and efficiency, age and, ultimately, the variation between energy intake (feed) and energy consumption (exercise, basal metabolic rate and body temperature maintenance, among others). Adipocytes store energy in the lipid droplets in the form of triacylglycerols when in an energy surplus condition and release them when energy demands in other tissues are high (Lonergan et al., 2019; Richard et al., 2000). Lipid composition on the other hand can be directly influenced by the composition of their diets, genotype, and level of fatness

in the carcass. For instance, and as previously shown in various studies, several local pig breeds, including the Portuguese AL and BI, present higher levels of MUFAs in the adipose tissue, particularly oleic acid, than selected pig genotypes (Charneca et al., 2019; Poklukar et al., 2020; Santos Silva et al., 2019).

Adipocytes can obtain and accumulate lipid molecules in their stores from two mechanisms, either from dietary consumption or through *de novo* lipogenesis. In the first one, adipocytes catalyse the release of free FAs from circulating triacylglycerol-rich transporters such as chylomicrons (produced in the small intestine enterocytes) and very low-density lipoproteins (produced in the liver), through the production of lipoprotein lipase (LPL). The liberated free FAs can then be used in the esterification of new triacylglycerol molecules and stored within the lipid droplets (Richard et al., 2000; Sylvers-Davie and Davies, 2021). For glycerol supplies, adipocytes rely on a conversion from circulating glucose, an uptake that can be boosted by insulin, in a process mediated by an insulin sensitive glucose transporter, GLUT4 (Stein and Litman, 2015). The second mechanism involves the endogenous synthesis of FAs in the adipocytes. In pigs, differently to what occurs in other livestock, the adipose tissue is the main location for lipid synthesis, with *de novo* lipogenesis providing over 80% of the total stored FAs (Kloareg et al., 2007). This process is characterized by the conversion of excess dietary carbohydrates and/or protein into FAs (palmitate) which can then be esterified to a glycerol backbone, assembling triacylglycerols to be stored. *De novo* lipogenesis can be divided in two major steps: carboxylation of acetyl CoA and the set of reactions mediated by the fatty acid synthase (FASN) multi enzymatic complex (Figure 5) (Richard et al., 2000). Before that occurs, the transported glucose is metabolized in the mitochondrion and is released as citrate, which is one of the main precursors for the production of acetyl-CoA, a reaction catalysed by ATP-citrate lyase (ACLY) that also produces oxaloacetate. The later can be transformed to malate through the action of malate dehydrogenase (MDH) and then to pyruvate to enter the glycolytic pathway. The other main precursor of this process is NADPH, which is predominantly obtained through the reversible oxidative decarboxylation of malate, a reaction catalysed by malic enzyme 1 (ME1) (Liang and Jiang, 2015). In the first and rate-limiting step of the *de novo* biosynthesis, the obtained acetyl-CoA undergo carboxylation, with the cost of ATP, to form malonyl-CoA, a reaction catalysed by acetyl-CoA carboxylase (ACACA). During the second step, malonyl CoA is repeatedly cycled to generate a long chain FA. Within each four-step cycle (comprising condensation, reduction, dehydration, and reduction, respectively) two carbon atoms are added to the acyl chain of the activated malonyl CoA group. The synthesis finishes after seven cycles, requires NADPH supply, and its action is regulated by the multifunctional enzyme complex FASN. The final end product of the *de novo* lipogenesis, palmitate, is a basic sixteen carbon saturated FA that can be used as a precursor molecule for the production of other FA types through elongation and desaturation steps. These steps can also be catalysed by other enzymatic complexes such as the ones coded by the elongation of long-chain FAs family member 6 (*ELOVL6*) and the stearoyl-CoA desaturase (*SCD*) genes, respectively (Ameer et al., 2014; Berg et al., 2015; Lehninger et al., 2008).

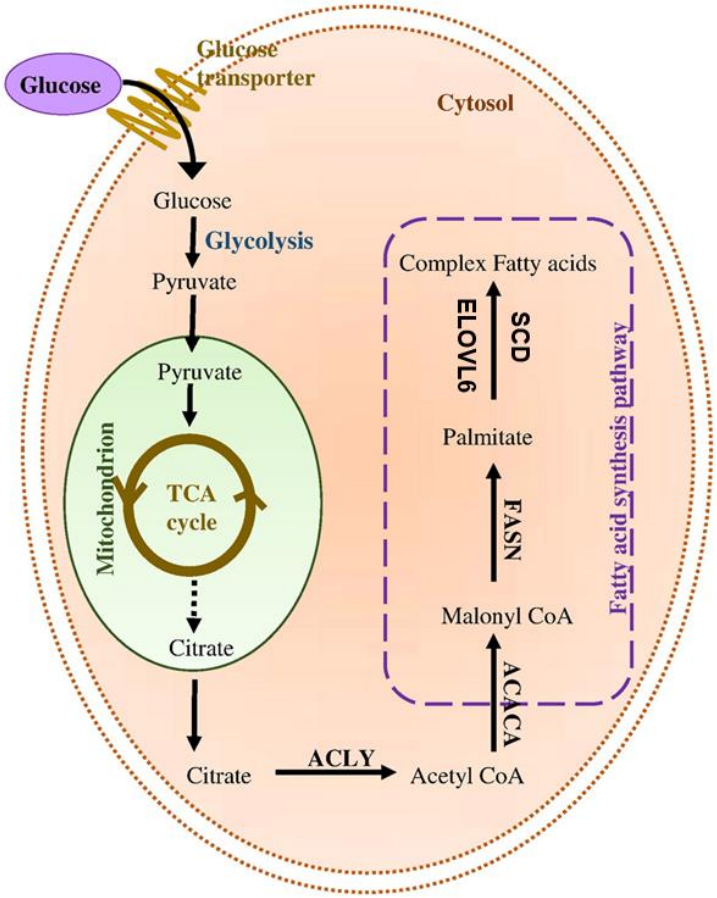

Figure 5. Summary of the endogenous biosynthesis of lipids from a glucose substrate. (Adapted from Ameer et al. (2014)).

At the other end of the spectrum regarding lipid metabolism is lipid catabolism which occurs when energy levels in the body are low or there is a high circumstantial energetic demand. This process can take place in all tissues but mainly occurs within the adipose tissue, where most energy supply is stored (Richard et al., 2000). Lipolysis includes three distinct steps: recruitment of free FAs, transport to the mitochondrial matrix, and beta-oxidation. The first step is characterized by the action of adipocyte lipases that sequentially hydrolyse stored triacylglycerols into free FAs and glycerol. These lipases can be activated through hormonal signals, such as epinephrine and glucagon, which also indirectly regulate perilipin proteins that restructure lipid droplets for an easier access by the lipases. Adipose triglyceride lipase (ATGL) is the first lipase in action and hydrolyses the release of one FA, then hormone sensitive lipase (HSL) can hydrolyse the resulting diacylglycerol to release a second FA. Finally, monoacylglycerol lipase (MAGL) completes the mobilization step by releasing the last FA and the glycerol backbone. The obtained FAs are not soluble in the circulatory bloodstream but bind into serum albumin that transports them to the target tissues, while the glycerol molecule can be metabolized in the liver

and participate in the glycolytic or gluconeogenic pathways. The second step comprises the activation and transport of these free FAs to the mitochondrial matrix, where oxidation of the acyl groups will occur. This is only required for long chain free FAs (more than 12 carbons) and the transport mechanism is mediated by a carnitine complex. Activation involves the formation of a thioester acyl CoA, using ATP and a CoA substrate, and still takes place on the outer mitochondrial membrane. Carnitine acyltransferase 1 (CPT1) catalyses the reaction where the acyl group present in the activated FA binds to the hydroxyl group of carnitine to form acyl carnitine, allowing the acyl carnitine ester to enter the mitochondrial matrix through an acyl carnitine translocase. Carnitine acyltransferase 2 (CPT2) catalyses the opposing reaction, allowing the carnitine group back to the cytoplasm where it can bind to another activated acyl group while a CoA group binds to the transported acyl group (Berg et al., 2015; Lehninger et al., 2008). Finally, when within the mitochondrial matrix, the fatty acyl CoA group can be oxidized in a four-step cycling process of oxidation by flavin adenine dinucleotide (FAD), hydration, oxidation by NAD^+ and thiolysis by a CoA group (Berg et al., 2015; Richard et al., 2000) (Figure 6).

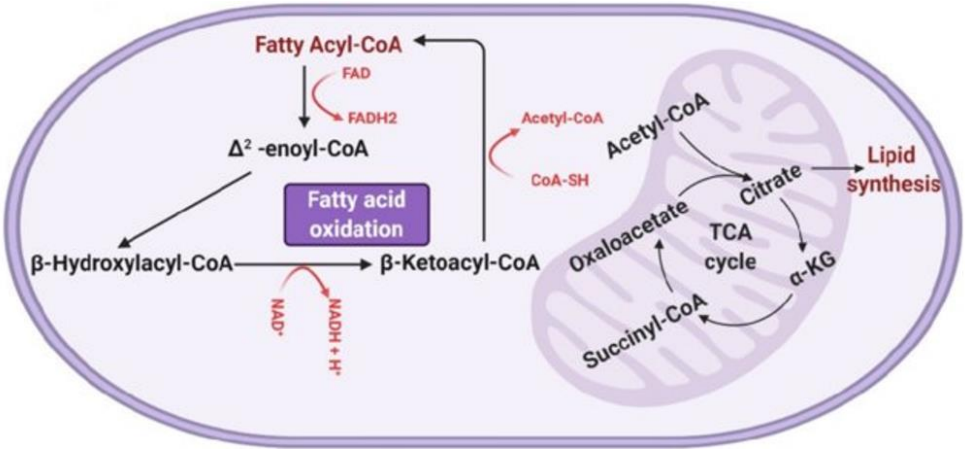

Figure 6. Summary of the beta oxidation process (Adapted from Caniglia et al. (2021)).

Each cycle removes two carbon atoms from the starting acyl CoA group, yielding in the process one molecule of $FADH_2$, NADPH and acetyl CoA. The acetyl CoA groups produced by each cycle can enter the Citric Acid Cycle and, just as those produced from glycolysis, be oxidized to CO_2, generating more electron carrier molecules as well as ATP from the respiratory (electron-transfer) chain (Lehninger et al., 2008).

1.4.2. Skeletal muscle tissue

Three types of muscle are present in pigs as well as in all mammals, namely: cardiac, smooth/involuntary, and skeletal/voluntary muscles. The basic units of the skeletal muscle are known as myocytes or muscle fibres. They present a cylindrical shape, contain various nucleus and mitochondria, form a striated pattern, and represent 90% of the muscle. These cells are enveloped together by layers of connective tissue (endomysium, fascia, perimysium, and epimysium) which support and protect the muscle fibres (Figure 7), allowing them to endure muscle contractions. Furthermore, each skeletal muscle portion also includes segments of nervous, vascular, and adipose tissues (up to 10%). These portions are linked to the bones through the tendons, collagen enriched connective tissues, and are essential for performing movements that are independently coordinated via electric signalling by the central nervous system (Listrat et al., 2016; NIH, 2019).

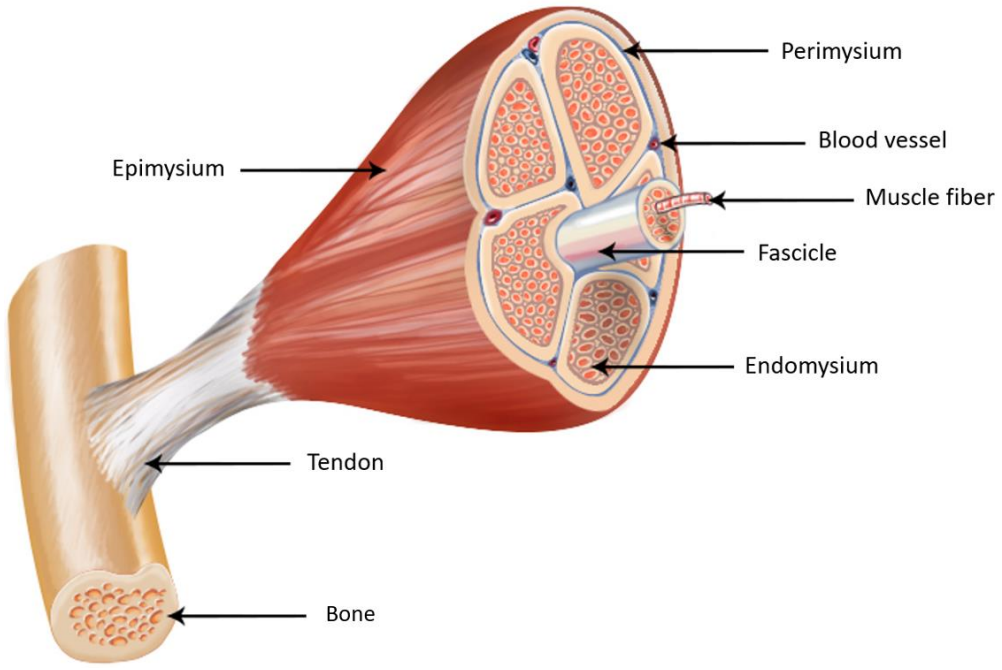

Figure 7. The general structure of skeletal muscle (Adapted from (NIH, 2019)).

Each muscle fibre contains myoglobin, an oxygen-binding protein, and glycogen within the sarcoplasm, which provides energy for the cell's activity. Also present within the sarcoplasm are elongated structural proteins known as myofibrils which extend along all the muscle fibre length. These are mainly composed of two kinds of contractile myofilaments, thick myosin protein filaments and thin actin protein filaments, both disposed in a sarcomere section (Woodhead and Craig, 2015). Sarcomeres constitute the basic unit of myofibrils, and these

include M lines in its middle portion that offer support and organize the myosin filaments into A bands. The Z disc structures, delineating the lateral borders of the sarcomere, are responsible for anchoring the thin filaments. Finally, the I bands are the regions of a striated muscle sarcomere that contains thin filaments, and therefore the less dense (Lange et al., 2020) (Figure 8).

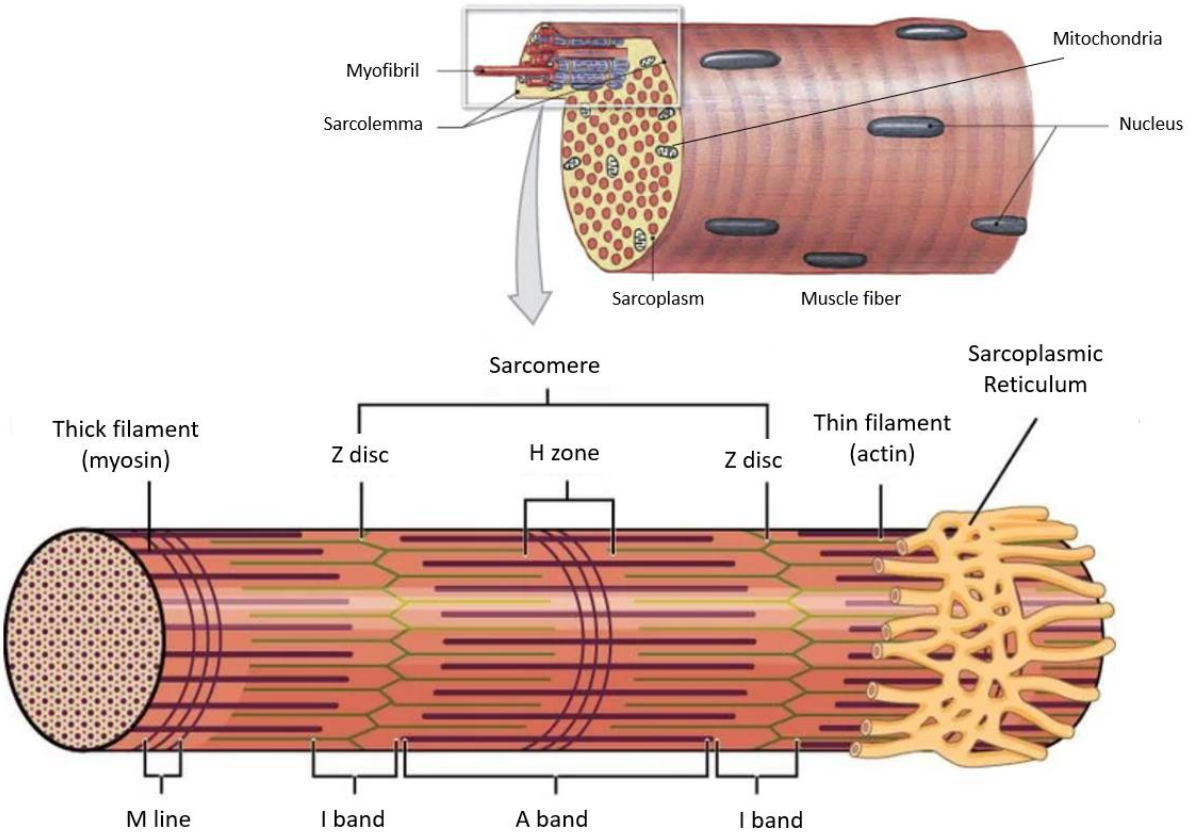

Figure 8. The structure of myofibrils within the muscle fibre (Adapted from Gollapudi et al. (2014); Udayangani (2017)).

Tubular shape myofibrils are parallelly arranged in a muscle cell and are responsible for the muscle contractions through the interaction between both types of filaments, in a reaction mediated by intracellular calcium (Ca^{2+}) concentrations, which are supplied by the sarcoplasmic reticulum. During contractions the actin myofilaments slide between the myosin filaments, reducing the distance between Z discs and shortening the sarcomeres, a process with an associated ATP cost (Dennis and Noakes, 2003; Woodhead and Craig, 2015). Furthermore, the presence of regulatory proteins troponin (TNNT family) and tropomyosin (TPM family) prevent the natural binding between myosin and actin filaments by exposing the binding sites only at specific calcium concentration levels (Krans, 2010).

In mammals, each muscle group presents different amounts of fibre types (Schiaffino and Reggiani, 2011). Fibre types in a muscle are intrinsically associated with meat quality and these are mostly classified according to their energy source and movement rate (Karlsson et al., 1999). The oxidative and glycolytic pathways are the two main ways for muscle cells to obtain energy. The first one is more efficient, but the second one allows for a faster muscle response, since oxygen availability is not limiting the speed of the catabolic reaction (Choe et al., 2008). Skeletal muscle fibres can be divided into two types, slow-twitch or type 1 and fast-twitch which include both 2A and 2B types. Type 1 muscle fibres are characterized for presenting more mitochondria and myoglobin content than 2A and 2B to fuel movement, granting them a darker appearance. These fibres are therefore known as red slow-twitch muscle fibres because they perform slow contractions sustained by an aerobic/ slow oxidative mitochondrial metabolism that do not change throughout their entire lifespan. Type 2A fibres are also known as red fast-twitch, they still feature a great ability for producing aerobic energy but can change their metabolism to anaerobic/glycolytic at specific times. These fibres also present less mitochondria than type 1, granting them a lighter appearance. Type 2B fibres, on the other hand, are known as white fast-twitch muscle fibres since they possess less myoglobin, mitochondria, and capillaries than the rest, but are richer in glycogen supplies. Fast twitch muscle fibres can generate low amounts of energy very fast through glycolytic metabolism, but fatigue at a much faster rate when compared to slow muscle fibres, making them ideal for short intense bursts (Schiaffino and Reggiani, 2011). Some authors also acknowledge the presence of other fibre types such as 2X, with intermediate characteristics between types 2A and 2B (e.g. Choe et al., 2008; Schiaffino and Reggiani, 2011; Schiaffino et al., 2015; Talbot and Maves, 2016). Furthermore, slow fibres are more efficient than fast in sequestering circulating glucose via GLUT4. It has been previously suggested that changes in fibre type distribution within the muscle group favouring fast twitch types are associated with lower insulin sensitivity and obesity (Schiaffino and Reggiani, 2011).

Skeletal muscle is a highly adaptable tissue, changing its phenotype regarding fibre size and composition (directional switch from 2B ↔ 2X ↔ 2A ↔ 1) through alterations in gene expression, which influence the occurring biochemical and metabolic processes. Furthermore, several environmental factors and physiological stimuli such as exercise, electrical stimulation, hypo- and hyperthyroidism can lead these changes to occur in either direction (Matsakas and Patel, 2009; Schiaffino and Reggiani, 2011). Muscle development can be established by either hyperplasia, an increase in the number of muscle fibres, or hypertrophy, an increase in the size of the already present muscle fibres. In mammals the total number of muscle fibres is genetically determined before birth with most postnatal development being associated with muscle hypertrophy, rather than hyperplasia, achievable by an increase in fibre length and width (Yang, 2014). Furthermore, the process of myogenesis in mammals has four distinct growing phases, namely embryonic, foetal, neonatal and adult, each displaying specific development traits and being subject of intensive research (Murphy and Kardon, 2011). It begins with the creation of precursor cells from the somites (segmental axial structures of the embryo) which can later differentiate into myoblasts. These myoblasts will proliferate and fuse to create primary muscle

fibres, encompassing various nucleus when stimulated via positive or negative growth factor molecules. Primary myofibers tend to be larger than secondary myofibers. Works in mice have previously revealed that during the prenatal and early postnatal stages of life, the development of myosin heavy chain embryonic (such as MYH3) and neonatal (such as MYH8) isoforms, which precedes the development of fast adult myosins, can account for over 95% of the total myosin heavy chain expression (Schiaffino et al., 2015). The same studies showed that after weaning the expression of these myosins dramatically decreases, with most skeletal muscle fibres presenting higher expression levels of myosins associated with fast adult fibre types 2A (MYH2), 2B (MYH4) and 2X (MYH1) rather than the slow type 1 (MYH7) (Matsakas and Patel, 2009; Schiaffino, 2018).

Directly influencing the occurrence of the myogenic events is the delicate but complex balance between anabolic and catabolic processes. When protein synthesis surpasses breakdown, also known as positive protein turnover, muscle growth can take place, involving a high cost of ATP, while when the breakdown of existing proteins surpasses protein synthesis, muscle degradation occurs (Yin et al., 2013). Muscle fibres can change their phenotype and metabolism, based on the nutritional contents of the provided diet, in order to optimize protein turnover. This response mechanism occurs through signal transduction, starting with an extracellular stimulus that interacts with receptors present at the surface of the target cells, activating signalling pathways that eventually lead to fibre remodelling by influencing gene expression. Nevertheless, when the stimulus is associated with a long-term energy restricted diet, most studies that address muscle growth claim a decrease in the observed muscle mass (Matsakas and Patel, 2009). Protein synthesis takes place in all tissues, however the positive feedback influence from feeding is quite clear on the skeletal muscle in pigs. Previously it has been demonstrated that, after a meal, the rise of circulating glucose, insulin, and amino acids enhances protein synthesis and, consequently, induce higher protein deposition. On the other hand, during protein degradation, ATP molecules are produced from the breaking of proteins into peptides and free amino acids, which can later be used as precursors for new protein synthesis. Furthermore, degradation of protein molecules contributes to the regulation of several physiological mechanisms, particularly in the conservation of structural integrity and in cellular signalling (Yin et al., 2013).

Most modern pig breeding programs still aim at muscle mass improvements in order to obtain more efficient and valuable carcasses. Skeletal muscle is the main component of consumed meat (Muirhead et al., 2013), and the *Longissimus lumborum* is one of the most economically relevant tissues in pigs and a reference muscle in meat quality studies due to its relative mass, and its variability in quality (Kauffman et al., 1990). This muscle is located in the lumbar region and is known for mostly containing fast twitch white muscle fibres, with low myoglobin content but high glycogen reserves, granting a fast anaerobic energy source to the cells. Meanwhile, muscles presenting a predominance of these fibres have previously been associated with a low water holding capacity and lighter colour (Su et al., 2013). However, the agreement between muscle fibre types and specific meat texture patterns is not clear in current literature (Lebedová et al., 2019). Fibre type composition can be influenced by genetic factors as

well as environmental ones, such as exercise, feeding, climate conditions, use of growth promoting substances, and others (Klont et al., 1998).

Historical selection in pigs determined a heavy genetic pressure to provide more lean muscle content and that may also have caused a shift in the composition of the muscle fibres. Selected leaner breeds tend to present bigger fibres with higher width and higher ratios of type 2 glycolytic fibres when compared to those of local unselected breeds. The latter may also be the cause of meat quality issues in some lean genotypes (Klont et al., 1998). Because *post-mortem* muscles no longer receive oxygen from the blood, they solely depend on their glycogen reserves and on anaerobic glycogenolysis and glycolysis, in a fruitless attempt to sustain their regular energetic levels. This process of degrading glycogen for energy leads to an overaccumulation of lactate and H^+ (Huff-Lonergan, 2010), causing the muscle pH to drop from 7.2 to 5.6 on average, with the normal range of ultimate pH in pork usually varying between 5.5 and 5.8 (Honikel, 1987). The decline in ultimate pH is associated with a decline in several meat quality attributes, some due to the denaturation of structural myocyte proteins, resulting in faded colour and lower water holding capacity for example. The *post-mortem* pH decline is also strongly linked to the occurrence of pale, soft and exudative (PSE) pork, an unwelcome attribute that yearly causes extensive economic losses to the pork industry (Lametsch et al., 2011; Scheffler and Gerrard, 2007). In leaner selected pig breeds with higher proportions of glycolytic myofibers, this issue reaches another level since the higher glycogen content leads to higher declines of pH in the meat. On the other hand and according to Bidner et al. (2004) at intermediate pH (5.4 - 6.0) the eating of the loin is most desirable and at high pH (above 6.2) unacceptable flavour intensity may increase.

Chapter 2

Growth, blood, carcass and meat quality traits from local pig breeds and their crosses – article #1

Martins, J.M.; Fialho, R.; Albuquerque, A.; Neves, J.; Freitas, A.; Nunes, J.T.; Charneca, R. Growth, blood, carcass and meat quality traits from local pig breeds and their crosses. animal 2020, 14, 636-647. https://doi.org/10.1017/S1751731119002222

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Note: The statistical analysis of the chemical composition and physical properties of the *Longissimus lumborum* tissue of AL and BI pig breeds slaughtered at ~150kg BW (n=9, for each breed) can be found in Appendix I.

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Growth, blood, carcass and meat quality traits from local pig breeds and their crosses

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*Contrary to intensive pig production, local pig breeds and their production systems are able to respond to the high criteria and expectations of modern society in regard to some environmental aspects, animal welfare, food quality and healthiness. This study proposes the recovery, study and use of a cross between two local breeds, contributing to animal biodiversity conservation and to the income of local pig producers. This work studied the growth performance and blood, carcass and meat quality traits of Alentejano (**AL**), Bísaro (**BI**) and Ribatejano (**RI**) (AL × BI, BI × AL) castrated male pigs. Raised outdoors, pigs were fed commercial diets ad libitum and killed at ~65 kg (trial 1, n = 10 from each genotype) and ~150 kg BW (trial 2, n = 9 from each genotype). In trial 1, AL and AL × BI attained slaughter weight later than BI and BI × AL pigs, with AL presenting lower average daily gains than the other genotypes (P < 0.001). Alentejano and RI pigs presented higher (P < 0.01) levels of plasma total protein than BI. Overall, carcass traits were affected by genotype, with length (P < 0.01), yield (P = 0.07) and lean cut proportions (P < 0.01) lower in AL than BI, and intermediate values for crossed pigs. Conversely, AL pigs presented higher fat cut proportion (P < 0.01), average backfat thickness (P < 0.001) and 'zwei punkte' fat depth (P < 0.01) than BI and RI pigs. Alentejano pigs also presented higher Longissimus lumborum (**LL**) intramuscular fat (P < 0.05), myoglobin content and ultimate pH (P < 0.01), but lower total collagen (P < 0.05), drip (P < 0.001) and cooking losses (P < 0.01), and shear force (P < 0.001) than all other genotypes. Finally, LL showed a more intense red colour in AL than in BI pigs. In trial 2, AL pigs confirmed to be a slow-growing obese breed with lower bone and lean cut proportions than BI, and higher LL intramuscular fat, richer colour, lower water loss and higher tenderness. In both trials, RI pigs grew faster, with higher lean and lower fat cut proportions and backfat thickness, and with overall LL characteristics comparable to those observed in AL pigs. This work demonstrates some clear differences between AL and BI breeds while showing that their crosses present intermediate characteristics in most studied traits. These data on RI pigs can be useful to breeders' associations and farmers in order to consider the use of these crosses as an option or complement to pure line breeding.*

Keywords: swine, Alentejano, Bísaro, Ribatejano, meat physicochemical characteristics

Implications

Local pig breed production chains and the growing market of their (very) high-quality products agree more with current consumers' environmental, animal welfare, food quality and healthiness concerns than conventional production systems. This study proposes the recovery, study and use of a cross between two local pig breeds (Alentejano and Bísaro), the Ribatejano pig. This additional production alternative could help to maintain or increase the pure breed populations, through a sustainable production of less expensive, high-quality meat and meat products, which could potentially increase the profit of (small to medium-scale) pig producers.

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Introduction

Nowadays, consumers select meat and meat products not only according to the (accessible) price and perceived eating quality, but also by their nutritional value and healthiness, animal welfare issues and the ethics underlying the production of meat, as well as the level of environmental impact caused by the production system (Font-i-Furnols and

Guerrero, 2014; Bergstra *et al.*, 2017). As a future challenge, it is necessary to increase the production of high-quality meat and meat products from pigs produced in sustainable systems and matching consumers' demands (Christensen *et al.*, 2008). European meat production systems based on local pigs generally give a more favourable response to these issues than intensive pig production. However, compared to conventional production systems, those based on local breeds are associated with slower growth rates, higher slaughter weights and fatter carcasses (Pugliese and Sirtori, 2012; Lebret *et al.*, 2014; Čandek-Potokar *et al.*, 2019). Therefore, several adaptations were introduced in the chain over the years to reduce costs and improve its economic viability, preserving the quality of the products, in many cases in compliance with the Protected Denomination of Origin (**PDO**) regulations. One of these adaptations was crossbreeding Alentejano/Iberian with Duroc pigs, to improve performance and productivity. On the other hand, local breed populations are generally composed of a small number of animals, and according to FAO (2011), animal biodiversity is vital to efficient and sustainable food production in the world and to meet the very different needs of human societies. Therefore, if local pig production chains could improve their performance and productivity by crossing local breeds instead of local with modern breeds, without compromising the quality of the final product, this could help to maintain or increase the local breed populations, leading to the conservation of animal biodiversity.

Alentejano (**AL**) and Bísaro (**BI**) are the two main local pig breeds from Portugal. Alentejano pig, with an extreme genetic closeness to the Iberian pig (Muñoz *et al.*, 2018), belongs to the Iberian type (Porter, 1993) and is characterised by low growth rate (except under 'montanheira' regime), and precociously high adipogenic activity (Neves *et al.*, 1996). Bísaro pig belongs to the Celtic type (Porter, 1993), presents poor growth (although higher than AL) and is a more muscular and less fatty breed (Santos e Silva *et al.*, 2000a). However, they are recognised for their high-quality meat and processed products (Santos e Silva *et al.*, 2000a; Freitas, 2014). Almost extinct in the 1980s (Santos e Silva *et al.*, 2000a), these two breeds are currently recovering, helped by the consumers' increased interest on traditional pig farming systems and sustainable production, animal welfare and meat and product quality issues. Nowadays these production systems (e.g., AL/Iberian pig production chain) represent an economic, ecological and social added value to their regions (Pugliese and Sirtori, 2012; Santos Silva, 2012). However, despite the mentioned recovery, these breeds are still considered threatened (AL) and rare (BI) breeds by official institutions (MAM, 2015).

Efforts to improve the growth performance of outdoor finishing pigs are continuously being made, and one way of improving it is through crossbreeding. Until the 1950s (Miranda do Vale, 1949), crosses between AL and BI breeds were a common practice and the animals, meat and products obtained were rather appreciated. Still, no data are available from these animals (Ribatejano pig, **RI**). The purpose of this study, framed in a European project focused on local pig breeds (TREASURE), was to evaluate the growth, carcass and meat quality of AL, BI and RI crosses (AL × BI and BI × AL) reared outdoors, fed commercial diets *ad libitum* and killed at ~65 and 150 kg BW. Productive variables, blood parameters, carcass, cuts as well as physicochemical traits of *Longissimus lumborum* (**LL**) were determined.

Materials and methods

Animals and experimental design

This work involved two trials with pure AL and BI pigs, and their reciprocal crosses, RI pigs (AL × BI and BI × AL), in four experimental groups. Eighty male pigs ($n = 20$ for each genotype) were surgically castrated at ~6 days of age. Pigs were raised in a traditional free-range system from 28.6 ± 0.5 kg BW (mean ± SEM) until ~65 kg (trial 1) and from ~65 to ~150 kg (trial 2). Animals were all-day-round in an outdoor park (~10 000 m^2) with shade provided by a zinc shed (~50 m^2) and dispersed trees, and were fed commercial diets. Composed of maize, soybeans, wheat, barley, rapeseed and animal fat, diets varied between 15.4 and 16.6 g/100 g CP, 12.4 and 12.9 g/100 g NDF, 4.5 and 5.1 g/100 g crude fat and 14.1 and 14.3 MJ/kg digestible energy (see Supplementary Material Table S1). In trial 1, diets were offered in group at an estimated *ad libitum* consumption (INRA, 1984) in a single daily meal (0900 h). In trial 2, pigs were individually fed, and diet refusals measured daily. Animals were fed at a weekly adjusted daily rate, between 1.6 (at 30 to 35 kg BW) and 4.0 kg (at 145 to 150 kg), and had free access to water. Average temperature, average minimal and maximal temperature, and average relative humidity were, respectively, 11.2°C, 6.5°C, 17.4°C, and 75.0% in trial 1 (January to April), and 21.9°C, 13.7°C, 31.1°C, and 56.3% in trial 2 (April to October).

At the end of trial 1, 10 animals per genotype were killed by CO_2 stunning and bleeding, at an average BW of 64.2 ± 0.3 kg. At the end of trial 2, nine animals per genotype were killed at an average BW of 150.6 ± 0.9 kg. Animals, killed at a commercial slaughterhouse, were fasted (~16 h) during lairage but had free access to water. Individual blood samples were collected during exsanguinations (10 ml EDTA tubes; Primavette, Kabe Labortechnik GmbH, Germany). Plasma obtained by centrifugation (2500×**g**, 4°C for 10 min; Fiberlite F21-8 × 50y rotor, Sorvall Lynx 4000; Thermo Scientific, Waltham, USA) was frozen (−80°C) (HFU 686 Basic; Heto, Brondby, Denmark) until analysis.

Plasma analyses

Plasma levels of total protein, urea, glucose, triacylglycerols and total cholesterol were determined by Advia® Chemistry enzymatic kits in an autoanalyzer (Advia® 1800; Siemens Healthcare Diagnostics Inc., Germany). Plasma cortisol concentrations were determined by the Immulite 2000 cortisol assay, using an IMMULITE 2000® autoanalyzer (DPC, Los

Angeles, CA, USA). Intra- and inter-assay CV are reported in Supplementary Material Table S2.

Carcass traits and tissue sampling

At slaughter, hot carcasses were weighed, and measures were made on the left half-carcass. Carcass length was measured from the anterior edge of the first cervical vertebrae to the posterior edge of the last lumbar vertebrae. Backfat thickness was averaged from two measurements, done between the last cervical and first thoracic vertebrae, and the last thoracic and first lumbar vertebrae. 'Zwei punkte' (**ZP**) fat and muscle depths were measured as the minimal fat (plus rind) depth over the muscle *Gluteus medius*, and the minimal muscle depth between the cranial end of the *Gluteus medius* and the dorsal part of the medullar canal, respectively. Commercial cuts were performed according to the Portuguese Norm (as described by Martins *et al.* (2012)), and their weights and those of belly and backfat were recorded. *Longissimus lumborum* samples were collected from the left-half carcasses, refrigerated (4°C) or vacuum-packaged and frozen (−20°C) for analyses.

Muscle tissue analyses

Longissimus lumborum ultimate pH (**pH_u**) at 24 h post-mortem was determined by a pH meter with a puncture electrode (LoT406-M6-DXK-S7/25; Mettler-Toledo GmbH, Germany).

EZ drip loss was determined on refrigerated LL samples (Christensen, 2003), and thawing and cooking losses were determined on LL chops (8 × 5 × 4 cm) which were weighed, vacuum-packed and frozen at −20°C until analysis. Thawing loss was obtained from samples thawed overnight (4°C) and reweighed. After thawing, samples were used to determine cooking losses. Samples were vacuum-packed, cooked in a water bath (70°C, 50 min; Heidolph Heizbad WB), cooled to room temperature and weighed (Lebret *et al.*, 2015). Shear force was measured perpendicular to the direction of fibres in rectangular cooked meat sections (1 × 1 × 3 cm) on a Texture Analyser TA HD Plus (Stable Micro Systems Ltd, Surrey, UK) equipped with a Warner-Bratzler V-shaped shear blade (1.2 mm thick) (Honikel, 1998). Averaged values determined from at least 8 (trial 1) and 12 sections (trial 2) of each LL sample were used for statistical analysis.

Total protein (total nitrogen × 6.25) was determined by the Dumas combustion method (method 992.15) (AOAC, 2011) with Leco FP-528 (Leco Corp., St. Joseph, MI, USA). Total intramuscular fat (**IMF**) was determined according to Folch *et al.* (1957), and myoglobin and total collagen as previously described (Martins *et al.*, 2012). Briefly, myoglobin content was determined in ground muscle samples (10 g) based on heme extraction with acetone and oxidation with hydrochloric acid, followed by spectrophotometric measurement (640 nm). Intramuscular collagen content was determined based on the extraction of hydroxyproline by hot hydrolysis in an acid medium, followed by spectrophotometric measurement (557 nm). Intra- and inter-assay CV of chemical analyses are reported in Supplementary Material Table S3.

Surface colour measurements of raw LL samples were recorded after blooming (30 min) with a CR-400 colorimeter (Konica Minolta Sensing Europe B.V., Nieuwegein, Netherlands) equipped with a D65 illuminant. *Commission internationale de l'éclairage* L^* (lightness), a^* (redness) and b^* (yellowness) values were obtained by averaging six random readings across muscle surface (as described by Martins *et al.* (2012)). Chroma ($C^* = (a^{*2}+b^{*2})^{1/2}$), hue angle ($H^\circ = \tan^{-1}(b^*/a^*)$) and saturation ($C^*/L^*$) were calculated.

Calculations and statistical analyses

Results are presented as mean ± rSD. All data were tested for normality by the Shapiro-Wilk test. Individual data of growth, plasma, carcass and meat quality traits were analysed by one-way ANOVA with genotype as the main effect. For the carcass data, hot carcass weight was included as a covariate in the model. The SPSS Statistics software (IBM SPSS Statistics for Windows, v24.0; IBM Corp., Armonk, NY, USA) was used for data analysis. Mean differences were considered significant when $P < 0.05$, and values between 0.05 and 0.10 were considered trends.

Results

Apart from one BI, which died during trial 1, all pigs remained in good health throughout the experimental period.

Trial 1: pigs killed at ~65 kg BW

Growth data. All experimental groups presented similar initial and final weights. However, AL and AL × BI pigs spent more time than BI and BI × AL ($P < 0.001$) to attain the slaughter weight, with AL pigs presenting lower average daily gain (**ADG**) ($P < 0.001$) than BI and RI crosses (AL × BI and BI × AL) (Table 1).

Fasting plasma parameters. Genotype did not influence plasma glucose, triacylglycerols, total cholesterol and cortisol levels of pigs. However, AL, AL × BI and BI × AL pigs presented higher ($P < 0.01$) levels of plasma total protein, while urea tended ($P = 0.07$) to be higher in AL × BI than in BI pigs (Table 2).

Carcass characteristics and cut proportions. Hot carcass weight was not significantly affected by genotype, but carcass length ($P < 0.01$), head proportion ($P < 0.05$) and carcass yield ($P = 0.07$) were lower in AL than BI pigs (Table 3). Overall, commercial yield ($P < 0.01$) and the proportions of lean cuts untrimmed shoulder ($P < 0.01$), loin ($P < 0.05$) and untrimmed ham ($P < 0.001$) were also lower in AL than in BI pigs, with RI crosses presenting intermediate values. Conversely, belly ($P < 0.05$), backfat proportions ($P < 0.001$) and therefore fat cut proportions ($P < 0.01$) were higher in AL than in BI and RI pigs. Therefore, lean-to-fat cut ratio was lower ($P < 0.001$) in AL pigs, followed by AL × BI, BI × AL and BI pigs. Average backfat thickness ($P < 0.001$),

Table 1 *Growth data of AL (n = 20), BI (n = 19), AL × BI (n = 20) and BI × AL (n = 20) pigs at ~65 kg BW*

	AL	BI	AL × BI	BI × AL	rSD	*P*-value[1]
Initial weight (kg)	28.4	29.0	27.4	29.8	4.0	0.29
Final weight (kg)	64.6	64.2	64.4	65.1	2.0	0.50
Days on trial	108.6^{a}	89.8^{b}	101.0^{a}	89.0^{b}	15.4	<0.001
Average daily gain (g/day)	333.6^{b}	396.5^{a}	374.5^{a}	401.5^{a}	45.7	<0.001

AL = Alentejano; BI = Bísaro.
[1]Values within a row with different superscripts differ significantly.

Table 2 *Plasma parameters of AL (n = 10), BI (n = 10), AL × BI (n = 10) and BI × AL (n = 10) pigs killed at ~65 kg BW*

	AL	BI	AL × BI	BI × AL	rSD	*P*-value[1]
Total protein (g/l)	74.3^{a}	69.9^{b}	72.5^{a}	72.7^{a}	2.2	0.002
Urea (mmol/l)	5.26	4.84	5.36	4.92	0.49	0.064
Glucose (mmol/l)	6.23	6.04	6.20	6.07	1.12	0.98
Triacylglycerols (mmol/l)	1.09	0.79	0.97	0.90	0.36	0.33
Total cholesterol (mmol/l)	2.84	2.87	2.84	2.85	0.35	0.99
Cortisol (nmol/l)	362.3	385.8	374.9	381.6	68.2	0.88

AL = Alentejano; BI = Bísaro.
[1]Values within a row with different superscripts differ significantly.

Table 3 *Carcass and cut trait data of AL (n = 10), BI (n = 10), AL × BI (n = 10) and BI × AL (n = 10) pigs killed at ~65 kg BW*

	AL	BI	AL × BI	BI × AL	rSD	*P*-value[1]
Hot carcass weight (kg)	47.0	48.0	48.1	47.8	1.7	0.47
Carcass length (cm)	71.8^{c}	82.1^{a}	75.4^{b}	76.0^{bc}	3.1	0.004
Head (%)	8.5^{b}	9.8^{a}	8.9^{b}	8.8^{ab}	0.6	0.018
Carcass yield (%)	73.3	75.2	74.9	73.9	1.6	0.068
Commercial yield (%)[2]	45.0^{c}	51.2^{a}	48.0^{b}	49.5^{ab}	1.4	0.002
Untrimmed shoulder	15.8^{c}	17.5^{a}	17.0^{ab}	16.6^{bc}	1.0	0.002
Loin	4.8^{b}	5.7^{a}	5.3^{ab}	5.4^{ab}	0.7	0.048
Untrimmed ham	24.0^{c}	27.8^{a}	25.9^{b}	26.0^{b}	0.9	<0.001
Tenderloin	0.63	0.53	0.63	0.61	0.08	0.36
Fat cuts (%)[3]	35.3^{a}	24.4^{c}	28.4^{b}	27.9^{b}	3.4	0.008
Belly	20.9^{a}	16.8^{b}	18.0^{b}	17.7^{b}	2.9	0.016
Backfat	14.4^{a}	7.6^{b}	10.3^{b}	10.2^{b}	2.9	<0.001
Bone cuts (%)[4]	15.3^{c}	19.4^{a}	16.8^{b}	17.1^{b}	1.6	0.001
Loin (bone-in, bladeless)	10.0^{c}	13.2^{a}	11.2^{b}	11.1^{bc}	1.3	0.002
Ribs	5.2	6.2	5.6	6.0	1.1	0.27
Lean-to-fat cut ratio	1.28^{c}	1.99^{a}	1.56^{b}	1.87^{a}	0.12	<0.001
Average backfat thickness (mm)[5]	38.1^{a}	21.1^{c}	32.5^{ab}	30.4^{b}	6.1	<0.001
Fatness: ZP[6] fat depth (mm)[7]	25.2^{a}	16.9^{b}	22.8^{a}	24.1^{a}	4.7	0.002
Leanness: ZP muscle depth (mm)[8]	48.2	47.6	48.7	49.1	1.3	0.34

AL = Alentejano; BI = Bísaro.
[1]Values within a row with different superscripts differ significantly.
[2]Percentage relative to carcass of the sum of untrimmed shoulder, loin, untrimmed ham and tenderloin.
[3]Percentage relative to carcass of the sum of belly and backfat cuts.
[4]Percentage relative to carcass of the sum of loin (bone-in, bladeless) and ribs.
[5]Average of measurements taken between last cervical and first thoracic vertebrae (first rib), and last thoracic and first lumbar vertebrae (last rib).
[6]'zwei punkte' = two points.
[7]Minimal fat depth (including rind) over *Gluteus medius*.
[8]Minimal muscle depth between the anterior extremity of *Gluteus medius* and the dorsal part of the medullar canal.

Table 4 *Chemical composition, pH, drip loss, thawing loss, cooking loss, Warner-Bratzler shear force and CIE colour values of* Longissimus lumborum *from AL (n = 10), BI (n = 10), AL × BI (n = 10) and BI × AL (n = 10) pigs slaughtered at ~65 kg BW*

	AL	BI	AL × BI	BI × AL	rSD	*P*-value[1]
Moisture (g/100 g)	73.1^{b}	74.7^{a}	73.6^{ab}	73.3^{b}	1.4	0.048
Total protein (g/100 g)	22.8	22.1	22.6	23.1	1.2	0.42
Total intramuscular fat (g/100 g)	6.7^{a}	5.5^{b}	5.9^{ab}	5.6^{b}	1.0	0.029
Myoglobin content (mg/g)	0.76^{a}	0.54^{b}	0.69^{a}	0.65^{ab}	0.12	0.007
Total collagen (mg/g DM)	13.9^{a}	17.1^{b}	15.3^{ab}	15.7^{ab}	2.3	0.037
pH (24 h post-mortem)	5.57^{a}	5.44^{b}	5.45^{b}	5.50^{b}	0.08	0.002
Drip loss (g/100 g)	1.35^{c}	3.76^{a}	2.24^{b}	2.74^{b}	0.66	<0.001
Thawing loss (%)	7.3	11.4	7.9	10.8	5.3	0.28
Cooking loss (%)	17.7^{b}	28.9^{a}	21.5^{b}	22.0^{b}	5.2	0.002
Warner-Bratzler shear force (N)	26.6^{b}	47.4^{a}	30.4^{b}	34.1^{b}	8.9	<0.001
Lightness (CIE *L**)	50.8^{c}	55.7^{a}	53.0^{b}	53.5^{b}	2.2	<0.001
Redness (CIE *a**)	10.3^{a}	7.8^{b}	10.3^{a}	10.2^{a}	1.0	<0.001
Yellowness (CIE *b**)	3.57	4.00	4.04	3.93	0.53	0.20
Chroma (*C**)	11.0^{a}	8.8^{b}	11.0^{a}	10.9^{a}	1.0	<0.001
Hue angle (*H°*)	19.1^{b}	27.3^{a}	21.5^{b}	21.1^{b}	2.9	<0.001
Saturation	0.22^{a}	0.16^{b}	0.21^{a}	0.21^{a}	0.02	<0.001

CIE = Commission internationale de l'éclairage; AL = Alentejano; BI = Bísaro.
[1]Values within a row with different superscripts differ significantly.

as well as ZP fat depth ($P < 0.01$), generally agreed with these results, presenting higher values in fatty AL than in leaner BI pigs. Finally, the bone cut proportion was lower in AL than in BI pigs, due to a lower ($P < 0.01$) loin (bone-in, bladeless) proportion (Table 3).

Muscle tissue analyses. Longissimus lumborum physico-chemical data were affected by genotype (Table 4). Moisture content was lower ($P < 0.05$) in AL and BI × AL than in BI pigs, but total protein was not different between genotypes. Total IMF, inversely related to the moisture content, was higher ($P < 0.05$) in AL than in BI and BI × AL pigs. Myoglobin content was higher ($P < 0.01$) in AL and AL × BI than in BI pigs, and total collagen was lower ($P < 0.05$) in AL than in BI pigs, with their crosses presenting intermediate values. Soluble collagen was not affected by genotype (Supplementary Material Table S4).

The pH_u values were higher ($P < 0.01$) in LL from AL than from BI and RI pigs (Table 4). *Longissimus lumborum* drip losses from AL pigs were the lowest, and those of BI pigs the highest, with their crosses presenting intermediate values ($P < 0.001$), and cooking losses from AL pigs and RI crosses were lower ($P < 0.01$) than those of BI pigs. The differences in thawing losses followed the same trend but did not attain statistical significance. Warner-Bratzler shear force (**WBSF**) values of LL from AL pigs were the lowest, but only statistically different ($P < 0.001$) from those of BI pigs (Table 4). Yet, LL texture profile analysis (**TPA**) was not affected by genotype (Supplementary Material Table S5).

Longissimus lumborum lightness (*L**) was lower ($P < 0.001$) on AL than BI pigs, again with their crosses presenting intermediate values. Redness (*a**) was higher ($P < 0.001$) on AL and RI than BI pigs. These results affected chroma (C*) and hue angle (H°) values, respectively higher and lower ($P < 0.001$) on AL and RI than BI pigs. Finally, LL saturation values were higher ($P < 0.001$) on AL and RI than BI pigs (Table 4).

Trial 2: pigs killed at ~150 kg BW

Growth data. All genotypes presented similar initial and final weights. When compared to AL and AL × BI, BI and BI × AL pigs presented a lower number of days on trial, but this difference did not attain statistical significance. Also, ADG only tended ($P = 0.06$) to be lower in AL × BI when compared to BI and BI × AL pigs, mainly due to a lower ADG ($P = 0.06$) in the second half of the trial (between 100 and 150 kg BW) (Table 5).

Fasting plasma parameters. Fasting plasma parameters influenced by genotype were total protein, triacylglycerols and total cholesterol. As observed at 65 kg, AL and AL × BI pigs presented higher ($P < 0.01$) levels of total protein than BI, but also than BI × AL pigs (Table 6). Triacylglycerols ($P = 0.08$) and total cholesterol levels ($P < 0.01$) were higher in AL than in BI pigs, with their crosses presenting intermediate values for triacylglycerols and similar values to those of AL for cholesterol. Finally, plasma cortisol levels tended to be lower ($P = 0.08$) in AL and AL × BI pigs and higher in BI pigs.

Carcass characteristics and cuts proportions. Hot carcass weight of pigs slaughtered at trial 2 was not significantly affected by genotype. As in trial 1, shorter carcasses ($P < 0.01$) were obtained from AL than BI pigs, with their crosses presenting intermediate lengths (Table 7). Head proportion was lower ($P < 0.001$) in AL and RI pigs. Carcass yield was higher ($P < 0.01$) and commercial yield lower ($P < 0.05$) in AL and AL × BI, when compared to BI and BI × AL pigs. Trimmed shoulder ($P < 0.01$), loin ($P < 0.05$), and untrimmed ($P < 0.001$) and trimmed ham proportions ($P < 0.01$) were

Table 5 *Growth data of AL (n = 9), BI (n = 9), AL × BI (n = 9) and BI × AL (n = 9) pigs slaughtered at ~150 kg BW*

	AL	BI	AL × BI	BI × AL	rSD	*P*-value
Initial weight (kg)	65.1	65.6	64.8	65.2	2.3	0.91
Final weight (kg)	152.7	150.0	147.2	152.4	5.6	0.16
Days on trial	146.1	135.3	150.0	137.8	16.7	0.22
Feed conversion ratio (kg/kg)	5.2	4.7	5.4	4.6	0.8	0.10
Feed conversion ratio 65 to 100 kg	4.7	4.1	4.6	4.3	0.6	0.13
Feed conversion ratio 100 to 150 kg	5.6	5.2	6.0	4.8	1.1	0.14
Average daily gain (g/day)	602.8	638.8	556.2	633.3	70.5	0.060
Average daily gain 65 to 100 kg	538.9	589.3	554.2	576.8	52.3	0.19
Average daily gain 100 to 150 kg	655.0	669.1	564.0	689.3	99.4	0.056

AL = Alentejano; BI = Bísaro.

Table 6 *Plasma parameters of AL (n = 9), BI (n = 9), AL × BI (n = 9) and BI × AL (n = 9) pigs slaughtered at ~150 kg BW*

	AL	BI	AL × BI	BI × AL	rSD	*P*-value[1]
Total protein (g/l)	78.0^{a}	73.3^{b}	78.7^{a}	73.2^{b}	4.0	0.008
Urea (mmol/l)	6.3	6.4	6.6	6.5	0.8	0.90
Glucose (mmol/l)	8.9	8.7	8.2	8.5	1.5	0.76
Triacylglycerols (mmol/l)	0.88^{a}	0.71^{b}	0.76^{ab}	0.81^{ab}	0.13	0.079
Total cholesterol (mmol/l)	2.55^{a}	2.06^{b}	2.45^{a}	2.39^{a}	0.26	0.002
Cortisol (nmol/l)	370.9	492.6	351.3	409.8	121.9	0.081

AL = Alentejano; BI = Bísaro.
[1]Values within a row with different superscripts differ significantly.

Table 7 *Carcass and cut trait data of AL (n = 9), BI (n = 9), AL × BI (n = 9) and BI × AL (n = 9) pigs slaughtered at ~150 kg BW*

	AL	BI	AL × BI	BI × AL	rSD	*P*-value[1]
Hot carcass weight (kg)	125.8	121.7	123.2	123.9	4.7	0.34
Carcass length (cm)	88.7^{c}	104.8^{a}	96.6^{b}	94.3^{bc}	4.4	0.002
Head (%)	6.4^{b}	7.4^{a}	6.6^{b}	6.2^{b}	0.5	<0.001
Carcass yield (%)	82.4^{ab}	81.1^{b}	83.7^{a}	81.3^{b}	1.3	0.002
Commercial yield (%)[2]	46.5^{b}	48.2^{a}	46.2^{b}	49.2^{a}	2.6	0.049
Untrimmed shoulder	20.5	19.0	19.4	19.6	1.8	0.39
Trimmed shoulder	10.7^{b}	13.1^{a}	11.2^{b}	10.5^{b}	1.5	0.002
Loin	3.64^{b}	4.61^{a}	4.56^{a}	4.36^{a}	0.70	0.042
Untrimmed ham	21.2^{b}	23.9^{a}	21.3^{b}	24.4^{a}	1.4	<0.001
Trimmed ham	12.2^{c}	15.9^{a}	13.6^{b}	12.8^{bc}	1.0	0.002
Tenderloin	0.86	0.94	0.96	0.84	0.17	0.44
Fat cuts (%)[3]	31.4^{a}	25.2^{c}	28.8^{b}	28.6^{b}	2.6	0.001
Belly	15.8^{a}	14.5^{b}	14.9^{b}	16.0^{a}	0.9	0.006
Backfat	11.2^{a}	6.8^{c}	9.4^{ab}	8.3^{bc}	2.4	0.004
Bone cuts (%)[4]	11.3^{c}	13.7^{a}	12.5^{b}	12.1^{bc}	0.8	<0.001
Loin (bone-in, bladeless)	8.0^{c}	10.3^{a}	9.2^{b}	8.8^{b}	0.5	<0.001
Ribs	3.32	3.41	3.29	3.25	0.56	0.97
Lean-to-fat cut ratio	1.50^{c}	1.93^{a}	1.62^{bc}	1.73^{ab}	0.20	0.001
Average backfat thickness (mm)[5]	76.2^{a}	43.0^{c}	61.2^{b}	62.5^{b}	2.2	<0.001
Fatness: ZP[6] fat depth (mm)[7]	64.3^{a}	33.8^{c}	54.9^{b}	60.2^{ab}	2.9	<0.001
Leanness: ZP muscle depth (mm)[8]	63.5	61.8	65.1	62.7	1.3	0.35

AL = Alentejano; BI = Bísaro.
[1]Values within a row with different superscripts differ significantly.
[2–8]See Table 3 footnotes.

Table 8 *Chemical composition, pH, drip loss, thawing loss, cooking loss and CIE colour values of Longissimus lumborum of AL (n = 9), BI (n = 9), AL × BI (n = 9) and BI × AL (n = 9) pigs slaughtered at ~150 kg BW*

	AL	BI	AL × BI	BI × AL	rSD	*P*-value[1]
Moisture (g/100 g)	71.0[b]	72.1[a]	72.0[a]	72.0[a]	0.9	0.025
Total protein (g/100 g)	23.6	23.0	23.0	23.0	0.7	0.10
Total intramuscular fat (g/100 g)	6.9[a]	6.0[b]	6.5[ab]	6.1[b]	0.8	0.044
Myoglobin content (mg/g)	0.86[a]	0.45[b]	0.61[ab]	0.58[ab]	0.32	0.048
Total collagen (mg/g DM)	13.1[c]	16.7[a]	15.9[ab]	14.2[bc]	1.9	0.002
pH (24 h post-mortem)	5.72[a]	5.49[b]	5.70[a]	5.58[b]	0.10	<0.001
pH fall (45 min to 24 h)	0.91[a]	0.72[b]	0.89[a]	0.91[a]		0.025
Drip loss (g/100 g)	0.59[b]	1.92[a]	0.57[b]	0.84[b]	0.35	<0.001
Thawing loss (g/100 g)	3.30	4.47	3.63	4.37	1.70	0.40
Cooking loss (g/100 g)	25.4	26.2	24.4	24.9	2.1	0.33
Warner-Bratzler shear force (N)	41.9	51.4	44.3	43.8	8.4	0.10
Lightness (CIE *L**)	45.1[b]	50.0[a]	46.1[b]	46.4[b]	2.4	<0.001
Redness (CIE *a**)	11.5	10.9	11.3	11.1	1.6	0.89
Yellowness (CIE *b**)	3.18	3.50	3.37	3.43	0.74	0.82
Chroma (*C*)	11.9	11.5	11.8	11.7	1.6	0.94
Hue angle (*H°*)	15.7	18.1	16.2	17.1	3.0	0.36
Saturation	0.27[a]	0.23[b]	0.26[ab]	0.25[ab]	0.03	0.048

CIE = Commission internationale de l'éclairage; AL = Alentejano; BI = Bísaro.
[1]Values within a row with different superscripts differ significantly.

lower in AL than in BI pigs, with overall intermediate values in their crosses. Primal cuts (the sum of trimmed shoulder, loin and trimmer ham) proportions in AL, BI, AL × BI and BI × AL pigs were respectively 26.2%, 33.2%, 29.3%, and 27.3%. As observed in trial 1, fat cuts, belly and backfat proportions, were overall higher ($P < 0.01$) in AL than in BI and RI crosses, leading to a lean-to-fat cuts ratio lower ($P < 0.01$) in AL than BI and BI × AL pigs. Average backfat thickness and ZP fat depth agreed with these results, presenting higher ($P < 0.001$) values in AL pigs. Finally, bone cuts proportion was again lower in AL than in BI pigs, due to a lower ($P < 0.001$) loin (bone-in, bladeless) proportion in AL pigs (Table 7).

Muscle tissue analyses. Longissimus lumborum physico-chemical data were also affected by genotype (Table 8). Moisture content was lower ($P < 0.05$) in AL than in all other genotypes, and total IMF higher ($P < 0.05$) in AL than in BI and BI × AL pigs. *Longissimus lumborum* myoglobin content was higher ($P < 0.05$) in AL than in BI pigs, and total collagen lower ($P < 0.01$), with their crosses presenting intermediate values.

Longissimus lumborum pH_u values were higher ($P < 0.001$) in AL and AL × BI than in BI and BI × AL pigs (Table 8). *Longissimus lumborum* drip loss values of AL and RI were lower ($P < 0.001$) than those of BI pigs, but thawing and cooking losses, although lower in AL than BI pigs, did not attain statistical significance. *Longissimus lumborum* WBSF values from AL pigs tended to be lower ($P = 0.10$) than those of BI pigs, with their crosses presenting intermediate values.

Longissimus lumborum lightness (*L**) values were lower ($P < 0.001$) on AL and RI than on BI pigs, and saturation values were higher ($P < 0.05$) on AL than BI pigs, with their crosses presenting intermediate values (Table 8).

Discussion

Despite an increase in interest on local breeds and their production systems, compared with industrial genotypes, AL and (mainly) BI have been scarcely studied. Moreover, available scientific data were obtained in trials with rearing and feeding conditions and weight/age at slaughter, among others, being reported with a high level of discrepancy or, in some cases, not specified. Yet, to improve local breed sustainability, it is important to get scientific assessment of their productivity and product quality, among other factors (Čandek-Potokar *et al.*, 2019). So, additional studies in controlled experimental environment and various production stages are needed, especially since data available from AL and BI pigs were mostly obtained on fattening animals (Neves *et al.*, 1996; Santos e Silva *et al.*, 2000a; Martins *et al.*, 2015). Additionally, these are the first data obtained from RI pigs.

To evaluate the growth, carcass characteristics and meat quality of AL, BI and RI pigs reared outdoors and fed commercial diets *ad libitum*, animals were slaughtered at ~65 and 150 kg BW. In commercial production, local pigs killed at lower weights are generally consumed as roasted pigs or as fresh meat, while heavier pigs are used in the production of high-quality traditional cured products (Santos

Silva, 2012; Freitas, 2014), both with PDO, PGI and/or TSG European certification.

Trial 1: pigs killed at ~65 kg BW

Pigs were killed at an average age of 186.2 days. Genotypes slower to attain slaughter weight were AL and AL × BI (about 18% and 12% slower, respectively), with AL pigs presenting lower ADG than the other genotypes. Globally, these findings agree with the poor growth rate of both local breeds (Neves *et al.*, 1996; Santos e Silva *et al.*, 2000a), and with the lower weight of AL compared to BI pigs slaughtered at the same age (Santos e Silva *et al.*, 2000a). Conversely, the low average temperature verified during trial may have led to a higher energy spent in thermoregulation and not on growth. In fact, and despite being in more extreme outdoor conditions (average 5°C), Bee *et al.* (2004) observed a lower ADG in outdoor-growing pigs compared to those growing in indoor facilities (average 22°C).

Levels of biochemical blood parameters may be used to monitor and predict health status at slaughter, genetic disease resistance, performance traits and meat quality (Lingaas *et al.*, 1992), but little research has been conducted to evaluate blood metabolites of AL pigs, and none in BI and RI pigs. On this trial, the plasma non-lipid parameters observed were within the normal physiological range of growing-finishing pigs of lean European breeds (Lingaas *et al.*, 1992; Merck, 2016). In the case of AL, they were similar to the ones reported in 40 to 80 kg 85% *ad libitum*-fed pigs (Martins *et al.*, 2007), with the exception of triacylglycerols, which were lower in these pigs. Nevertheless, differences in plasma metabolites were observed between genotypes. Total protein was higher in AL and RI than in BI pigs, suggesting a higher protein accretion in leaner BI pigs. This highly energetic process (Wu *et al.*, 2007) may have contributed to the lower ($P \pm 0.05$) plasma levels of glucose (−3%) and triacylglycerols (−27.5%) in BI compared to AL pigs. A strong tendency ($P = 0.06$) for lower levels of plasma urea nitrogen in BI pigs suggests that they utilised nitrogen more efficiently for growth than AL and AL × BI genotypes. In fact, blood urea nitrogen reduction is generally associated with an increase in N utilisation efficiency and lean tissue growth (Coma *et al.*, 1995).

Alentejano pig is a medium-sized breed with a light bone structure (Freitas, 2014), while BI have tall, long and narrow bodies, long legs and considerable proportions of skin and bone (Santos e Silva *et al.*, 2000a). Recently, the c. 748C allele of the germ cell nuclear factor (*NR6A1*) gene, associated with shorter carcasses, was detected in AL but not in BI pigs (0.15 *v.* 0.00 frequencies, respectively) (Muñoz *et al.*, 2018). This was confirmed in our trial by the 12.5% shorter carcass length of AL compared to BI pigs. Alentejano head and bone cut proportions were also 13.3% and 21.1% lower, confirming this genotype morphological characteristics. As to RI pigs, both crosses presented intermediate values between AL and BI. Alentejano pigs also present carcasses with low lean and high fat cut proportions (Freitas, 2014), while in BI pigs, lean cuts predominate (Santos e Silva *et al.*, 2000a). Besides the polygenic nature of fatness traits, LEPR c.1987T allele, systematically associated with higher fatness, is almost fixed in the obese AL, compared to the leaner BI breed (0.98 *v.* 0.26 frequencies, respectively) (Muñoz *et al.*, 2018). These characteristics, generally present in pigs slaughtered at ±100 kg, were already observed in our growing animals, killed at 65 kg. Charneca and Nunes (2013) reported that, compared to Large-White × Landrace piglets, AL piglets presented lower DNA concentration in *L. dorsi*, which may indicate a lower number of muscle cells. According to Rehfeldt and Kuhn (2006), hypertrophy of muscle cells is enhanced when its number is lower, the limit of muscle cell growth is attained sooner, and energy is directed sooner to fat deposition. This could partly explain the higher fat content of AL pigs' carcasses, which presented 12.1% lower commercial yield and 44.7% higher fat cut proportions compared to BI. These differences were observed in the proportions of untrimmed shoulder, loin and untrimmed ham (−9.7%, −15.8% and −13.7%, respectively), and of belly and backfat (+24.4% and +89.5%, respectively) cuts. In another trial where AL and BI pigs were compared, these differences were also observed in pigs slaughtered at 96 to 105 kg (Santos e Silva *et al.*, 2000a). Overall, proportions of the different lean and fat cuts obtained at this trial are between those reported for outdoor Iberian pigs of 56 and 76.6 kg BW (Mayoral *et al.*, 1999) and agree with those from 85% *ad libitum*-fed AL with 100 kg (Martins *et al.*, 2012). As for BI pigs, the cut proportions were similar to lower to the ones from 85% *ad libitum*-fed BI with 105 kg (Santos e Silva *et al.*, 2000a). Once again, RI crosses presented intermediate values,

but in the case of fat cuts, these values were not significantly different from those of BI pigs. These differences led to a 35.7% lower lean-to-fat cut ratio, and an 80.6% and 49.1% higher backfat thickness and ZP fat depth, respectively, in AL compared to BI pigs.

Moisture and total protein from LL were close to slightly lower than those reported for 85% *ad libitum*-fed AL pigs with 100 kg (Martins *et al.*, 2015) and BI pigs (Leite *et al.*, 2015), but total IMF tended to be higher in our AL and BI pigs, probably due to different feeding conditions. Moreover, LL total IMF in the fatty AL genotype was 21.8% higher than in BI, and 19.6% higher than in BI × AL, confirming the precocious high adipogenic activity of AL pigs. Intramuscular fat is strongly influenced by genotype, and meat with a high content of IMF has better sensory properties and higher juiciness and palatability than meat with a low fat content (Fernandez *et al.*, 1999; Hocquette *et al.*, 2010). These characteristics are improved if the IMF content in LL increases above ~2.5% (Fernandez *et al.*, 1999), a limit that was surpassed in the studied genotypes.

Longissimus lumborum myoglobin content was higher in AL and AL × BI than in BI pigs, which could be a genotype characteristic, as previously suggested (Honikel, 1998). The skeletal muscle connective tissue network, predominantly consisting of collagen proteins, is a contributing factor to

the meat's texture (Gregory and Grandin, 1998). The total collagen content detected in LL of AL pigs was similar to the values reported in outdoor Iberian pigs with 76.6 kg BW (Mayoral *et al.*, 1999). However, the total collagen content was 18.7% lower in LL from AL than BI pigs, with RI crosses presenting intermediate values. This difference suggests a more tender meat in AL pigs, agreeing with the 43.9% lower WBSF values registered in LL samples of that genotype, compared to BI. Conversely, the IMF content in LL fibres affects cut resistance, with higher fat corresponding to lower shear force values (Essén-Gustavsson *et al.*, 1994), as observed in AL pigs. Our data suggest that AL and RI presented higher meat tenderness than BI pigs, the most important factor for the perceived sensory quality of pork (Van Oeckel *et al.*, 1999).

The pH_u normal range in pork varies between 5.5 and 5.8 (Honikel, 1987). This parameter has a direct influence on meat technological properties, such as water loss, colour, tenderness and flavour (Gregory and Grandin, 1998; Bidner *et al.*, 2004). On this trial, LL from AL pigs presented pH_u values similar to those reported by Martins *et al.* (2012) for AL, and BI and AL × BI values slightly below the lower limit of the normal pH_u range and to the values observed in 60 to 80 kg BI pigs (pH_u 5.6) (Leite *et al.*, 2015). This suggests a higher LL glycogen content in BI and AL × BI pigs, positively correlated with lower pH values (Bidner *et al.*, 2004).

Water loss by drip is an important factor in the loss of saleable product and pork quality. It may influence its nutritive value through the loss of soluble proteins, vitamins and minerals, and also colour traits, texture, flavour, odour and succulence, among others. This loss may be explained by the exhaustion of muscle ATP, leading to the establishment of irreversible crosses between actin and myosin proteins with a reduction of the myofibrillar space, and/or by a pH decrease to the protein isoelectric point, reducing the negative charges of the filaments and therefore the repulsive electrostatic forces (reviewed by Huff-Lonergan and Lonergan, 2005). Partial denaturation of the myosin head at a low pH may explain a large part of the shrinkage in myofibrillar lattice spacing (Honikel, 1998; Huff-Lonergan and Lonergan, 2005). Finally, water loss may also be influenced by the content and distribution of IMF, with higher values of IMF associated with decreases in the moisture diffusivity coefficient (Muriel *et al.*, 2004). In our trial, drip loss was 64.1% lower in LL from AL than BI pigs. These differences agree with the higher IMF and pH_u values observed in AL pigs, and therefore lower passive exudation. Meanwhile, this water loss was significantly reduced in RI crosses, compared to BI pigs. Water loss by thawing and cooking were also 36% and 38.8% lower in AL than in BI pigs, although the former did not attain statistical significance. A 23% cooking loss was previously recorded in LL from 60 to 80 kg BI pigs (Leite *et al.*, 2015).

Colour, the main parameter used for the consumer's critical first impression of meat, largely depends on myoglobin concentration, its chemical form and the physical state of meat (pH values, protein state and denaturation degree, water loss, among others) (Honikel, 1998). The LL values reported in the literature for colour traits (L^*, a^* and b^*) vary widely between breeds (e.g. 44 to 58, 5 to 14, and 4 to 9, respectively) (Muriel *et al.*, 2004; Martins *et al.*, 2012; Lebret *et al.*, 2015). Overall, LL values reported in this trial are within those ranges. Nevertheless, LL from AL pigs showed the lowest levels of L^* and $H°$ and the highest levels of a^* and saturation, which agrees with the higher levels of myoglobin and pH_u in that genotype. Therefore, compared to those from BI, LL from AL pigs presented a more intense red colour, with higher C^* and lower $H°$ values, an important characteristic to the consumer (Muriel *et al.*, 2004). According to Bidner *et al.* (2004), the paleness of pork is inversely proportional to pH, and BI pigs presented the lower pH_u of the genotypes studied. Conversely, the higher L^* values observed in LL from BI pigs could be partially explained by a higher drip loss obtained on that genotype, since surface water scatters more light and is positively correlated to lightness (Brewer *et al.*, 2001). L^* values of RI crosses were intermediate between those of pure genotypes. However, a^*, C^* and $H°$ values were not significantly different from those of AL pigs, suggestive of a more intense red colour of RI meat compared to BI meat.

Trial 2: pigs slaughtered at ~150 kg BW

On this trial, pigs were killed at an average age of 353.6 days. Alentejano and AL × BI pigs were slower to attain slaughter weight, but this difference was not significant as in trial 1, showing that growth rate was similar in fattening AL and BI pigs. The same happened with feed conversion ratio (although averages of AL plus AL × BI were ~14% higher than those of BI plus BI × AL pigs) and ADG (~12.5% lower in AL × BI than the average of BI plus BI × AL pigs), even though these traits tended to be statistically significant ($P = 0.10$ and 0.06, respectively). As expected, growth rates in this trial (at either 100 and/or 150 kg BW) were lower than those observed in modern genotypes, reaching daily gains >1000 g in optimal conditions of intensive systems (Čandek-Potokar *et al.*, 2019).

Overall, the plasma non-lipid parameters from pigs slaughtered at 150 kg BW were within the normal physiological range of growing-finishing pigs of lean European breeds (Lingaas *et al.*, 1992, Merck, 2016). In the case of AL pigs, these values were higher than those reported in pigs with 110 kg and fed a commercial diet at 85% *ad libitum* (Martins *et al.*, 2007). Interestingly, total cholesterol was lower in this trial (2.55 *v.* 3.04 mmol/l), probably due to the effect of exercise (Carey, 1997), since pigs in the 2007 trial were in individual pens and these were outdoors. Plasma total protein was higher in AL and AL × BI than in BI and BI × AL pigs. These results suggest a higher protein deposition in BI pigs, with this highly demanding energetic process leading to a lower plasma level of triacylglycerols (−19.3%) in BI compared to AL pigs. Also, genetically obese pigs have higher blood triglycerides and cholesterol concentrations than lean pigs (McNeel *et al.*, 2000), contributing to the differences in those blood constituents between AL and BI pigs. Finally, plasma cortisol levels were, respectively, 24.7% and 28.7% lower in AL and AL × BI than in BI pigs,

suggestive of a lower response to environmental stressors, and/or ante-mortem events and operations (reviewed by Faucitano (1998)). Outdoor systems are potentially more comfortable and provide a more enriched environment, more space and opportunity for pigs to exercise, but they have some disadvantages, such as a greater risk of heat stress. Since pigs do not pant or sweat, they use wallowing and evaporation as main heat loss mechanisms (Gregory and Grandin, 1998). In this trial, visual observation revealed an important wallowing behaviour in BI, a breed originating from the cooler northern regions of Portugal, in the last 3 months of trial, where 87% of the daily maximal temperatures were ≥30°C, and 50% ≥35°C. This agrees with the plasma cortisol levels, which increase in response to heat as a stressor (Gregory and Grandin, 1998).

Compared with BI pigs, AL pig's light bone structure and compact body was confirmed in trial 2, with a 15.4% shorter carcass length, 13.5% lower head and 17.5% lower bone cut proportion (−22.3% in loin (bone-in, bladeless)). Also, bone cuts in AL pigs declined 26.1% between 65 and 150 kg BW, confirming that they develop more precociously than lean and fatty cuts (Dobao *et al.*, 1987). Carcass yield increases with fat carcass content, since in pigs most of the fat is deposited in the carcass (subcutaneous and intermuscular) (Serrano, 2008). Hence, AL and AL × BI pigs presented higher carcass yields than BI and BI × AL pigs, even though only significant between AL × BI, and BI and BI × AL pigs. As to lean and fat cuts, AL pigs presented 3.5% lower commercial yield and 24.6% higher fat cut proportions than BI, confirming this breed's high capacity for fat deposition and lower genetic potential for lean gain (Santos e Silva *et al.*, 2000a; Freitas, 2014). These differences were observed in the proportions of trimmed shoulder, loin, and untrimmed and trimmed ham (−18.3%, −21%, −11.3% and −23.3%, respectively), and of belly and backfat (+9% and +64.7%) cuts. Curiously, this latter genotype differences were more pronounced in the growing than in the fattening period: +44.7% of fat cut proportion and +80.6% of backfat thickness in the former, and +24.6% and +77.2% in the latter period, for AL and BI pigs, respectively. The proportion of the different cuts detected in our trial are close to those reported for Iberian pigs with 140 kg BW *ad libitum*-fed concentrates (Dobao *et al.*, 1987). As to the lean cut proportion, values were similar to those obtained in 'montanheira' AL pigs with 150 to 160 kg (Grave, 2015), even though the fat cut proportions were lower, probably due to the highly energetic acorns consumed by the latter animals. As for BI, cut proportions were similar to the ones from pigs with 105 and 150 kg BW (Santos e Silva *et al.*, 2000b). Genotype differences led to a 22.3% lower lean-to-fat cut ratio, and a 77.2% and 90.2% higher backfat thickness and ZP fat depth, respectively, in AL compared to BI pigs, with their crosses presenting overall intermediate values. Finally, compared with heavy pigs from modern genotypes (Lebret *et al.*, 2014; Dalla Bona *et al.*, 2016), these local breeds exhibit lower growth rate, lower carcass lean content and higher carcass fatness and IMF content.

Longissimus lumborum moisture content was lower, but total IMF was 15% higher in AL than in BI and BI × AL pigs, suggesting better sensory properties and higher juiciness and palatability (Hocquette *et al.*, 2010) of LL from the fatty AL genotype. Compared to the IMF content observed in 65 kg AL pigs, we noticed an almost neglectable increase at 150 kg (6.7 to 6.9 g/100 g, respectively), as previously observed in Iberian pigs (Mayoral *et al.*, 1999). Nevertheless, when calculating the fat weight deposited in muscle ((muscle weight × %IMF)/100) at 65 and 150 kg, the values were 72.8 and 158.0 g, respectively. *Longissimus lumborum* moisture and total protein values were similar to those reported for *ad libitum*-fed castrated Iberian pigs with 145 kg (Serrano, 2008) and castrated male and female BI pigs with 150 kg (Santos e Silva *et al.*, 2000b). Meanwhile, LL IMF of AL pigs was lower than the one observed in 'montanheira' AL pigs slaughtered at 150 to 160 kg (Grave, 2015), probably due to the different feeding conditions and age at slaughter. *Longissimus lumborum* IMF of BI pigs was higher (at 65 and 150 kg) than the ones previously reported (Santos e Silva *et al.*, 2000a, Leite *et al.*, 2015), and the different feeding regimes, and age/weight at slaughter (well above the age of sexual maturity), could have contributed to these differences. Also, BI pigs from this trial were reared outdoors, with a certain amount of physical activity that enhances IMF diffusion within the fibres and allows high IMF deposition (Gago, 1989).

Myoglobin content was again higher in AL than in BI pigs, confirming the genotype effect on this parameter. *Longissimus lumborum* myoglobin content in AL pigs increased 13.2% from 65 to 150 kg BW (186 to 354 days of age), but curiously the content in BI pigs decreased 16.7%, suggesting that throughout animal life, pork gains a more intense colour in AL, as previously observed in Iberian pigs (Mayoral *et al.*, 1999), but seems to become paler in BI pigs.

At 150 kg BW, LL total collagen content of AL pigs was similar to the one of outdoor Iberian pigs with 152.8 kg BW (Mayoral *et al.*, 1999). Yet, total collagen was 21.6% lower in AL than BI pigs, which could contribute to a higher tenderness of AL pork. When instrumentally measured, LL tenderness of 150 kg AL pigs only tended ($P = 0.10$) to present lower shear force values compared with BI pigs. In fact, WBSF was ~44% lower in LL samples of AL pigs at 65 kg, but only 18.5% lower at 150 kg. One of the possible explanations for this difference is related to LL IMF content in these two genotypes at 150 kg. Higher IMF values generally correspond to lower shear force values (Essén-Gustavsson *et al.*, 1994), but compared to BI, at 150 kg AL pigs presented a 15% higher IMF content, whereas at 65 kg, that difference was 25%. Therefore, although with lower levels of total collagen at both weights, AL pigs showed a less expressive IMF difference in LL samples at 150 kg, leading to the WBSF values obtained. Furthermore, ageing animals present an increase in the number of stable bonds between collagen molecules, increasing its insolubility, which, with other parameters not measured in this trial such as proteolysis and myofibrillar fragmentation, may also play a role in determining tenderness (Huff-Lonergan and Lonergan, 2005).

The pH values obtained are within the normal range for pH_u in LL (Honikel, 1987), but, once again, AL pigs presented higher pH_u values than those from BI. This suggests, as observed in lean breeds with a high content of fast muscle fibres (IIb) and high glycolytic capacity, that BI pigs presented a higher muscle glycogen content, positively correlated with lower pH values (Bidner *et al.*, 2004).

Longissimus lumborum drip loss was 69.3% lower in AL than BI pigs. Since water loss may be influenced by pH (Huff-Lonergan and Lonergan, 2005) and the content and distribution of IMF (Muriel *et al.*, 2004) among other factors, the higher pH_u and IMF observed in LL from AL pigs could have contributed to their lower passive exudation.

Genotype effects on colour were less expressive than the ones detected during growth. *Longissimus lumborum* from AL pigs still presented lower levels of L^* and higher levels of saturation than BI pigs, but a^* and H° values, although respectively higher and lower in AL, did not attain statistical significance. Contrary to what was observed at 65 kg, a 91% significantly higher myoglobin content in LL of AL pigs was not reflected on significantly higher a^* values. However, sensory redness correlates highly with L^* value, while only moderately with H° and a^* values (Brewer *et al.*, 2001). On the other hand, as observed at 65 kg, higher L^* values measured in LL from BI pigs could also be partly explained by the higher drip loss observed on that genotype. Once more, crosses presented overall intermediate values between those of pure genotypes.

In conclusion, data obtained on trial 1 show that until 65 kg BW, AL pigs are a slow-growing obese breed with lower bone and lean cut proportions than BI pigs. However, AL pigs presented LL muscle with a higher IMF, richer colour, higher tenderness and lower water loss (passive and by cooking), features rather appreciated by the consumer and related to a higher quality of the meat (Lebret *et al.*, 2015). Ribatejano pigs presented intermediate features between the fatter AL and the leaner BI genotypes. They grew faster, presented higher lean and lower fat cut proportions and backfat thickness, as well as LL with a slightly lower IMF, identical rich colour and tenderness, and slightly higher (passive) to similar (by cooking) water loss than AL pigs. These characteristics detected in growing pigs were overall identical in the fattening ones (trial 2). The results of the present study on RI pigs regarding loin, shoulder and ham weights, as well as LL overall characteristics, suggest their potential to produce heavier high-quality meat and dry-cured products, which merit future investigations. Alentejano × Bísaro crossbreeds, more easily obtained in the north (BI homeland), could improve meat and meat product quality compared to the ones obtained with pure BI pigs. On the other hand, the BI × AL crossbreed, more easily obtained in the south (AL homeland), could lead to better commercial yield and primal cut weights, compared to those obtained from pure AL pigs, without significantly jeopardising quality issues. Finally, this potential use of RI crosses by pig farmers could enhance their revenue, improve the sustainability of the production system and contribute to maintaining or increasing local pig populations.

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Declaration of interest

The authors declare no conflicts of interest.

Ethics statement

All animals were reared and slaughtered in compliance with the regulations and ethical guidelines of the Portuguese Animal Nutrition and Welfare Commission (DGAV, Portugal) following the 2010/63/EU Directive.

Software and data repository resources

Data are not deposited in an official repository.

Supplementary material

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Chapter 3

Portuguese Local Pig Breeds: Genotype Effects on Meat and Fat Quality Traits – article #2

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Note: The statistical analysis of the chemical composition and physical properties of the *semimembranosus*, *gluteus medius* and dorsal subcutaneous fat tissues of AL and BI pig breeds slaughtered at ~150kg BW (n=9, for each breed) can be found in Appendix I.

animals

MDPI

Article

Portuguese Local Pig Breeds: Genotype Effects on Meat and Fat Quality Traits

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Simple Summary: Local breeds are generally associated with slower growth rates, higher slaughter weights, and fatter carcasses due to genetic and rearing system characteristics. When compared to intensive pig production systems, those based on European local breeds generally provide a more favourable response to the required increase in the production of high-quality pork and pork products in sustainable chains, meeting consumer demands. Reducing costs and improving the economic viability of production systems while preserving the quality of the products obtained is of vital importance. In this work, we propose that Portuguese local pig production chains could improve their performance and productivity without compromising the quality of the final product by crossing local breeds instead of crossing with modern breeds. This could help to maintain or increase local breed populations, supporting conservation of animal biodiversity.

Abstract: This work investigated the contribution of cross-breeding between two local Portuguese pig breeds to the conservation of animal biodiversity and income of local pig producers. Quality traits of semimembranosus (SM), gluteus medius (GM) and dorsal subcutaneous fat (DSF) were studied in Alentejano (AL), Bísaro (BI), AL × BI, and BI × AL (Ribatejano—RI) castrated male pigs. Pigs were reared outdoors, fed ad libitum, and slaughtered at ~65 (trial 1) and 150 kg BW (trial 2). In trial 1, AL pigs showed higher SM intramuscular fat, lower total collagen, and higher soluble collagen than BI pigs, while AL × BI and BI × AL pigs showed intermediate (NS) values. AL, AL × BI, and BI × AL pigs showed higher SM myoglobin content, and AL a more intense red colour than BI pigs. Finally, AL, AL × BI, and BI × AL showed higher total lipids in DSF than BI pigs. In trial 2, SM and DSF results were similar to those obtained in trial 1. In GM, AL and BI × AL showed higher intramuscular fat than BI and AL × BI pigs, while AL, AL × BI and BI × AL showed lower total collagen content than BI pigs. In conclusion, these results suggest that RI crosses are a productive alternative, with overall muscle and DSF traits statistically not different between AL × BI and BI × AL, and similar to those observed in AL pigs.

Keywords: swine; Alentejano; Bísaro; Ribatejano; meat quality; dorsal subcutaneous fat

1. Introduction

The increasing demand for pork products is leading to an international effort to save traditional pig breeds and develop new breeds [1]. In Portugal, the main local pig breeds are the Alentejano (AL), an Iberian type breed [2] with an extreme genetic closeness to the Iberian pig [3], and the Bísaro (BI) pig, from the Celtic type [2]. The AL pig is characterised by a low growth rate (except under "montanheira" regime) and precociously high adipogenic activity [4]. The BI pig presents a poor growth (although higher than AL), little backfat (although higher than that of industrial genotypes), and a high proportion of skin and bone [5]. These environmentally well-adapted local breeds are less productive than modern improved genotypes, and their production chains depend mainly on the marketing of meat, and fermented and dry-cured meat products with highly valued sensory characteristics [5–7]. Almost extinct in the 1980s [5,8], these two breeds and their traditional systems have recovered since the 1990s [6], driven by consumer concerns about animal welfare, sustainable production, and meat and meat product quality issues. Although still threatened (AL) and rare (BI) breeds [9], they currently have a high ecological, economic and social importance in their production regions [6,10,11]. Increased yields and reduced costs on these productive systems is a continuous challenge, and crossbreeding is one way of achieving it.

AL and BI breeds homelands, in the South and North of Portugal respectively [8], have contact zones in the Tagus River region. In these contact zones (e.g., Ribatejo region), crosses between the two breeds were common until the 1950s [12] and the meat and meat products obtained were highly appreciated. However, there are no data available regarding these crosses, popularly called Ribatejano (RI) pigs. This study, included in the TREASURE project dedicated to European local pig breeds, was the first to collect and analyse carcass, pork and fat data from crossbred RI pigs, using AL and BI pure animals as controls. It represents a potential new management strategy for these breeds' production chains, while attending to societal demands for environment preservation, sustainable local agro-economy, as well as to consumers demands for quality and healthiness on regional pork products. The recovery and commercial use of these crosses could also help to preserve the pure breed populations, maintaining animal biodiversity, essential for an efficient and sustainable world food production and to meet the different needs of modern human societies [13]. In fact, loss of biodiversity may lead to an impaired response to changing environments.

Following a previous work where growth, carcass traits and loin data were presented [14], this study evaluates meat and fat quality from AL, BI, and AL × BI and BI × AL (RI crosses) pigs, reared outdoors, fed ad libitum, and slaughtered at ~65 and 150 kg BW. Physicochemical traits of semimembranosus (SM), gluteus medius (GM), and dorsal subcutaneous fat (DSF) were determined.

2. Materials and Methods

2.1. Animals and Experimental Design

Experimental procedures and animal care were performed in compliance with the ethical guidelines and regulations of the Portuguese Animal Nutrition and Welfare Commission (DGAV—Directorate-General for Food and Veterinary, Portugal), following the 2010/63/EU Directive.

Composed by two sequential trials, this work had four experimental groups, with pure Alentejano (AL) and Bísaro (BI) pigs and their reciprocal crosses, AL × BI and BI × AL (Ribatejano (RI) pigs, the common name of these crossbred). Male pigs (n = 20 for each of the 4 genotypes) surgically castrated within the 1st week of age were reared outdoors from 28.6 ± 0.5 kg BW (mean ± SEM) until ~65 kg (trial 1) and from 65.2 ± 0.4 kg to ~150 kg (trial 2). In trial 1, pigs were group-fed with commercial diets (15.5–16.6 g/100 g crude protein, 12.4–12.7 g/100 g NDF, 4.5–5 g/100 g total lipids and 14.1–14.3 MJ/kg DE—Supplementary Table S1) at estimated ad libitum consumption [15], in a single daily meal (09:00 h). In trial 2, pigs were individually fed with commercial diets (15.4–16.6 g/100 g crude protein, 12.4–12.9 g/100 g NDF, 4.7–5 g/100 g total lipids and 14.1–14.3 MJ/kg DE—Table S1) and diet refusals were measured daily. All animals had free access to water and were weighed every fortnight.

Temperature and relative humidity data were registered throughout the experimental work. Average temperature, average minimal and maximal temperature, and average relative humidity were, respectively, 11.2, 6.5, 17.4 °C, and 75.0% in trial 1 (January–April), and 21.9, 13.7, 31.1 °C, and 56.3% in trial 2 (April–October).

Slaughtered at a commercial slaughterhouse in three batches per slaughter weight, animals had free access to water but were fasted for ~16 h during lairage. Ten animals from each genotype were slaughtered at the end of trial 1 (average BW of 64.2 ± 0.3 kg) and nine animals at the end of trial 2 (average of 150.6 ± 0.9 kg BW) by exsanguination following CO_2 stunning. Commercially reared local pigs are slaughtered at lighter weights for consumption as fresh meat or roasted pigs, or at heavier weights for the production of high quality traditional fermented and cured products [6,16] with PDO or PGI European certification.

2.2. Muscle and Adipose Tissue Sampling

Samples of SM muscle and of DSF were obtained from the left half carcasses at ~65 and 150 kg BW, while samples of GM muscle were collected only at ~150 kg. Samples were vacuum packaged and frozen (−20 °C) until analysis.

2.3. Muscles and Adipose Tissue Analyses

Leg (SM and GM) muscles represent a cut of greatest economic value and mass. Muscles ultimate pH (pH_u) was determined 24 h post-mortem by a pH-meter with a puncture electrode (LoT406-M6-DXK-S7/25, Mettler-Toledo GmbH, Germany).

Moisture was determined in muscles and DSF according to ISO-1442 [17]. Total nitrogen from muscle samples was determined in a Leco FP-528 (Leco Corp., St. Joseph, MI, USA) by the Dumas combustion method (method 992.15) and from DSF samples by the Kjeldahl method (method 928.08) [18]. Total protein content was estimated as total nitrogen × 6.25. Total lipids were determined in muscles according to Folch, et al. [19] and in DSF by Soxhlet extraction (method 991.36) [18]. Muscles myoglobin and total collagen were determined as previously described [20] and soluble collagen according to Hill [21].

Surface colour measurements [22] of raw SM and GM samples were recorded with a CR-400 colorimeter (Konica Minolta Sensing Europe B.V., Nieuwegein, Netherlands) with a D65 illuminant, after blooming for 30 min. Individual CIE (Commission Internationale de l'Éclairage) L^* (lightness), a^* (redness) and b^* (yellowness) values were averaged out of six random readings across muscle surface. The same procedure (without blooming) was applied to DSF samples. Chroma ($C^* = \sqrt{(a^{*2} + b^{*2})}$), hue angle ($H = tan^{-1}(b^*/a^*)$), and saturation ($C^*/L^*$) were calculated.

2.4. Statistical Analysis

All data were tested for normality by the Shapiro-Wilk test. Results are presented as mean ± rSD. Data were analysed by one-way analysis of variance (ANOVA) with SPSS Statistics software (IBM SPSS Statistics for Windows, v24.0, IBM Corp., Armonk, NY, USA). Mean differences were considered statistically significant when $p < 0.05$, and p values between 0.05 and 0.10 were considered trends.

3. Results

3.1. Trial 1: Pigs Slaughtered at ~65 kg BW

Pigs were slaughtered at an average age of 186.2 ± 2.9 days.

3.1.1. Muscle Tissue Analyses

SM physico-chemical data were affected by genotype (Table 1). Moisture was lower ($p < 0.05$) in AL and AL × BI than in BI pigs, while total intramuscular fat (IMF) was higher ($p < 0.05$) in AL than in

BI pigs. Myoglobin content was higher ($p < 0.01$) in AL pigs and Ribatejano (RI) crosses than in BI pigs, whereas total collagen was lower ($p < 0.05$) in AL than in BI pigs, with their crosses showing intermediate values. Regarding soluble collagen, as a % of total collagen, values were higher ($p < 0.05$) in SM from AL than BI pigs. SM pH_u values were not affected by genotype, but significant differences were observed in colour parameters (Table 1). Lightness (L^*) was lower and redness (a^*) higher ($p < 0.05$) on AL than BI pigs, again with their crosses showing intermediate values. These results affected hue angle (H°) and saturation, respectively lower ($p < 0.01$) and higher ($p < 0.05$) in AL than BI pigs (Table 1).

Table 1. Chemical composition, pH, and CIE colour values of *Semimembranosus* muscle from Alentejano (AL), Bísaro (BI), AL × BI and BI × AL pigs slaughtered at ~65 kg BW ($n = 10$ for each genotype).

Traits	AL	BI	AL × BI	BI × AL	rSD	*p*-Values
Moisture (g/100 g)	71.8 ^{b}	74.0 ^{a}	72.2 ^{b}	72.8 ^{a,b}	1.9	0.049
Total protein (g/100 g)	23.8	22.8	23.3	23.2	1.1	0.272
Total intramuscular fat (g/100 g)	5.9 ^{a}	4.7 ^{b}	5.3 ^{a,b}	5.2 ^{a,b}	1.0	0.040
Myoglobin content (mg/g)	0.42 ^{a}	0.18 ^{b}	0.40 ^{a}	0.33 ^{a}	0.13	0.002
Total collagen (mg/g DM)	15.7 ^{b}	19.0 ^{a}	17.2 ^{a,b}	17.7 ^{a,b}	1.9	0.010
Soluble collagen (% total collagen)	11.5 ^{a}	9.2 ^{b}	10.0 ^{a,b}	10.0 ^{a,b}	2.1	0.044
pH (24 h *post-mortem*)	5.42	5.52	5.48	5.50	0.14	0.468
Lightness (Cie L^*)	43.0 ^{b}	46.6 ^{a}	45.1 ^{a,b}	44.8 ^{a,b}	2.9	0.043
Redness (Cie a^*)	13.5 ^{a}	11.7 ^{b}	12.1 ^{a,b}	13.1 ^{a,b}	1.5	0.049
Yellowness (Cie b^*)	6.6	7.1	6.8	7.0	0.8	0.568
Chroma (C*)	15.1	13.8	13.9	14.8	1.6	0.214
Hue angle (H°)	26.3 ^{c}	31.4 ^{a}	29.5 ^{a,b}	28.3 ^{b,c}	2.8	0.004
Saturation	0.36 ^{a}	0.30 ^{b}	0.31 ^{a,b}	0.33 ^{a,b}	0.05	0.046

CIE—Commission Internationale de l'Éclairage. AL × BI and BI × AL represent the reciprocal crosses of the commonly known Ribatejano pig. DM—Dry matter. ^{a,b,c} Values in the same row with different superscript letters are significantly different ($p < 0.05$).

3.1.2. Adipose Tissue Analyses

Chemical composition of DSF was also affected by genotype (Table 2). Moisture content was lower ($p < 0.001$) in AL than in BI pigs, with RI crosses showing intermediate values. Total lipids, inversely related to moisture content, were higher ($p < 0.001$) in AL and RI crosses than in BI pigs (Table 2).

Table 2. Chemical composition, and CIE colour values of dorsal subcutaneous fat from Alentejano (AL), Bísaro (BI), AL × BI and BI × AL pigs slaughtered at ~65 kg BW ($n = 10$ for each genotype).

Traits	AL	BI	AL × BI	BI × AL	rSD	*p*-Values
Moisture (g/100 g)	7.3 ^{c}	11.0 ^{a}	8.5 ^{b,c}	9.0 ^{b}	1.4	<0.001
Total protein (g/100 g)	1.42	1.59	1.42	1.48	0.32	0.811
Total lipids (g/100 g)	85.0 ^{a}	71.5 ^{b}	81.4 ^{a}	81.6 ^{a}	4.6	<0.001
Lightness (Cie L^*)	82.3	80.7	81.1	80.6	2.5	0.459
Redness (Cie a^*)	2.91	3.50	3.18	3.29	0.6	0.257
Yellowness (Cie b^*)	4.84	5.17	4.92	4.97	1.1	0.937
Chroma (C*)	5.7	6.2	5.9	6.0	1.2	0.788
Hue angle (H°)	58.3	55.9	56.7	56.4	4.3	0.551
Saturation	0.07	0.08	0.07	0.07	0.01	0.590

AL × BI and BI × AL represent the reciprocal crosses of the commonly known Ribatejano pig. ^{a,b,c} Values in the same row with different superscript letters are significantly different ($p < 0.05$).

3.2. Trial 2: Pigs Slaughtered at ~150 kg BW

Pigs were slaughtered at an average age of 353.6 ± 2.6 days.

3.2.1. Muscle Tissues Analyses

Physicochemical data from SM samples were less affected by genotype in the fattening period. IMF was higher in AL and BI × AL than in BI pigs, but this difference did not attain statistical significance (Table 3). Total collagen was lower ($p < 0.05$) in AL pigs and RI crosses than in BI pigs, with soluble collagen (% total collagen) following the opposite trend without attaining statistical difference. SM pH_u was higher ($p < 0.05$) in AL than in BI pigs (Table 3). Regarding colour, genotype only tended to affect lightness (L^*) ($p = 0.067$) and yellowness (b^*) ($p = 0.059$) values, and therefore hue angle (H°) ($p < 0.05$) was lower in AL and AL × BI than in BI pigs (Table 3).

Table 3. Chemical composition, pH, and CIE colour values of *Semimembranosus* muscle from Alentejano (AL), Bísaro (BI), AL × BI and BI × AL pigs slaughtered at ~150 kg BW ($n = 9$ for each genotype).

Traits	AL	BI	AL × BI	BI × AL	rSD	*p*-Values
Moisture (g/100 g)	73.7	73.7	73.6	73.5	0.9	0.968
Total protein (g/100 g)	22.4	22.8	22.7	22.3	0.7	0.410
Total intramuscular fat (g/100 g)	5.0	4.5	4.6	5.1	0.7	0.326
Myoglobin content (mg/g)	1.93	1.75	1.82	1.80	0.36	0.786
Total collagen (mg/g DM)	15.5 ^{b}	20.7 ^{a}	17.1 ^{b}	17.0 ^{b}	3.5	0.029
Soluble collagen (% total collagen)	8.2	6.8	7.4	7.8	2.1	0.518
pH (24 h *post-mortem*)	5.76 ^{a}	5.51 ^{b}	5.67 ^{a,b}	5.66 ^{a,b}	0.16	0.027
pH fall (45min to 24 h)	0.55	0.65	0.65	0.56	0.29	0.833
Lightness (Cie L^*)	35.4	38.4	35.0	35.6	2.7	0.067
Redness (Cie a^*)	14.5	14.3	14.0	15.0	1.3	0.434
Yellowness (Cie b^*)	6.7	8.0	6.5	8.0	1.4	0.059
Chroma (C*)	16.0	16.5	15.5	17.0	1.6	0.241
Hue angle (H°)	24.6 ^{b}	29.1 ^{a}	24.5 ^{b}	27.8 ^{a,b}	3.8	0.039
Saturation	0.46	0.43	0.44	0.48	0.04	0.102

AL × BI and BI × AL represent the reciprocal crosses of the commonly known Ribatejano pig. ^{a,b} Values in the same row with different superscript letters are significantly different ($p < 0.05$).

IMF from GM muscle was higher ($p < 0.01$) in AL and BI × AL than in BI and AL × BI pigs, while total collagen was lower ($p < 0.01$) in AL pigs and RI crosses than in BI pigs. However, soluble collagen (% total collagen) and colour parameters of GM were not affected by genotype (Table 4).

Table 4. Chemical composition, pH, and CIE colour values of *Gluteus medius* muscle from Alentejano (AL), Bísaro (BI), AL × BI and BI × AL pigs slaughtered at ~150 kg BW ($n = 9$ for each genotype).

Traits	AL	BI	AL × BI	BI × AL	rSD	*p*-Values
Moisture (g/100 g)	69.9	70.6	70.2	69.9	1.2	0.529
Total protein (g/100 g)	21.7	22.4	22.2	21.8	1.3	0.580
Total intramuscular fat (g/100 g)	9.0 ^{a}	6.2 ^{b}	7.0 ^{b}	8.7 ^{a}	1.6	0.002
Myoglobin content (mg/g)	1.63	1.34	1.38	1.56	0.32	0.205
Total collagen (mg/g DM)	15.2 ^{b}	17.9 ^{a}	15.7 ^{b}	15.3 ^{b}	1.4	0.002
Soluble collagen (% total collagen)	8.5	8.8	8.7	8.6	1.6	0.982
pH (24 h *post-mortem*)	5.62	5.58	5.65	5.61	0.14	0.748
pH fall (45min to 24 h)	0.95	0.89	0.72	0.87	0.28	0.274
Lightness (Cie L^*)	39.2	40.3	38.7	39.5	2.9	0.702
Redness (Cie a^*)	12.3	12.2	11.8	13.0	1.5	0.344
Yellowness (Cie b^*)	5.5	5.8	5.4	6.5	1.4	0.387
Chroma (C*)	13.4	13.6	13.0	14.6	1.8	0.333
Hue angle (H°)	23.9	25.1	24.3	25.9	3.4	0.807
Saturation	0.34	0.33	0.33	0.36	0.04	0.329

AL × BI and BI × AL represent the reciprocal crosses of the commonly known Ribatejano pig. ^{a,b} Values in the same row with different superscript letters are significantly different ($p < 0.05$).

3.2.2. Adipose Tissue Analyses

Chemical data from DSF were affected by genotype, with total protein lower ($p < 0.001$) in AL pigs and RI crosses than in BI pigs, and total lipids higher ($p < 0.05$) in AL than in BI and AL × BI pigs (Table 5). Finally, DSF colour parameters were also influenced by genotype. Redness (a^*), chroma (C*), and saturation were lower ($p < 0.05$) in AL than in BI pigs, while hue angle (H°) was higher ($p = 0.05$), with RI crosses showing intermediate values.

Table 5. Chemical composition and CIE colour values of dorsal subcutaneous fat from Alentejano (AL), Bísaro (BI), AL × BI and BI × AL pigs slaughtered at ~150 kg BW ($n = 9$ for each genotype).

Traits	AL	BI	AL × BI	BI × AL	rSD	*p*-Values
Moisture (g/100 g)	5.1	5.8	5.6	5.4	0.9	0.519
Total protein (g/100 g)	0.91 ^{b}	1.29 ^{a}	0.98 ^{b}	0.94 ^{b}	0.14	<0.001
Total lipids (g/100 g)	88.9 ^{a}	83.7 ^{c}	85.5 ^{b,c}	87.1 ^{a,b}	3.3	0.012
Lightness (Cie *L**)	79.3	79.1	79.5	78.8	1.1	0.495
Redness (Cie *a**)	2.25 ^{b}	3.36 ^{a}	2.70 ^{a,b}	2.70 ^{a,b}	0.9	0.042
Yellowness (Cie *b**)	4.47	4.91	4.36	4.69	0.6	0.291
Chroma (C*)	5.0 ^{b}	6.0 ^{a}	5.2 ^{a,b}	5.5^{ab}	0.9	0.048
Hue angle (H°)	63.3 ^{a}	56.4 ^{b}	59.0 ^{a,b}	60.7 ^{a,b}	6.2	0.049
Saturation	0.06 ^{b}	0.08 ^{a}	0.07 ^{a,b}	0.07 ^{a,b}	0.01	0.050

AL × BI and BI × AL represent the reciprocal crosses of the commonly known Ribatejano pig. ^{a,b,c} Values in the same row with different superscript letters are significantly different ($p < 0.05$).

4. Discussion

Sustainability and animal welfare policies are increasingly being adopted by the food industry in response to consumer demands. These changes can help strengthen pork niche markets and broaden the target audience for small farmers practicing outdoor swine production [23]. However, farmers and researchers must find a way to improve productivity and product quality, and scientifically support product differentiation [23,24]. One way to improve the performance of outdoor finishing pigs is through crossbreeding. AL and (mainly) BI genotypes are not sufficiently studied, namely in the case of muscles other than longissimus lumborum. In addition, currently available information was obtained in trials with very different or not even described rearing and feeding conditions, as well as age/slaughter weights, among other aspects. Therefore, additional studies are required to evaluate different production stages, in controlled experimental environments.

In order to evaluate meat and fat quality from AL and BI pigs, as well as their reciprocal crosses reared outdoors and fed ad libitum with commercial diets, animals were slaughtered at ~65 and 150 kg BW.

4.1. Trial 1: Pigs Slaughtered at ~65 kg BW

Growth and carcass data from this trial were previously presented and discussed by Martins et al. [14]. Briefly, AL pigs had a shorter carcass length and lower bone cuts weight than BI, while Ribatejano (RI) crosses showed intermediate values. AL pigs also showed low lean and high fat cuts proportions, while in BI pigs lean cut proportions were more important. This agrees with the presence in both genotypes of the LEPR c.1987T allele, usually associated with higher fatness, that is almost fixed in the fatty AL when compared to the leaner BI pig breed (0.98 vs. 0.26 frequencies, respectively) [3]. The lower lean and higher fat cuts of the carcasses from AL pigs led to a 12.1% lower commercial yield and 44.7% higher fat cuts proportion than those observed for BI. These differences were due to changes in untrimmed shoulder (−9.7%), loin (−15.8%) and untrimmed ham (−13.7%), and in belly (+24.4%) and backfat (+89.5%) cuts. Meanwhile, although RI crosses showed overall intermediate values, their fat cuts proportions were not significantly different from those of BI pigs. These differences led to

lower lean-to-fat cuts ratio and higher backfat thickness and ZP ("Zwei punkte") fat depth in AL than in BI pigs [14].

Muscle physicochemical traits were affected by genotype. In SM, IMF content was 25.5% higher in AL than in BI pigs, confirming the precociously high adipogenic activity in the AL pig [4]. Strongly influenced by genotype, IMF is positively correlated with the sensory properties, juiciness and palatability of meat [25–27]. Meanwhile, compared to IMF values observed in 100 kg AL pigs fed at 85% ad libitum [20,28], and BI pigs [29], those observed in our trial were slightly higher in AL and similar in BI. The differences in the AL breed results, were probably due to different feeding conditions used in both trials.

Myoglobin content has been suggested as a genotype-related characteristic [30]. In our trial, myoglobin showed higher values in the SM muscle from AL pigs and RI crosses when compared to BI pigs. On the other hand, SM total and soluble collagen were respectively 17.4% lower and 25% higher in AL than BI pigs, with RI crosses showing intermediate values. Collagen proteins are the predominant constituents of skeletal muscle connective tissue network and a contributing factor to meat's texture [27,31]. Likewise, IMF content affects muscle cut resistance, with higher fat corresponding to lower shear force values [27,32]. Therefore, differences observed in IMF and collagen content of SM suggest a more tender meat in growing AL pigs, and tenderness is described as the most important factor for the perceived sensory quality of pork [33]. This trend was also observed in the longissimus lumborum (LL) samples from these animals [14]. Still, total collagen values were higher in SM than in LL, confirming that hindquarter muscles used for locomotion such as biceps femoris, semimembranosus, and semitendinosus, are inherently tougher than support muscles such as longissimus lumborum [27]. Finally, the pH_u values, not affected by genotype, were close to the lower value of the normal range in pork, which varies between 5.5 and 5.8 [34].

Consumer's critical first impression of meat depends mainly on colour, which is in turn largely associated to myoglobin concentration and its chemical form. Other factors, such as the physical state of meat, including pH value, protein state, denaturation degree, and water loss, are also important [30]. SM values for colour coordinates in the literature are scarce and vary widely among breeds (e.g., 36–57, 3–17, and 4–15, for L^*, a^*, and b^*, respectively) [7,20,29,35–37]. Values observed in this trial are within the above-mentioned ranges. In our trial, the SM lowest levels of L^* and H° and the highest levels of a^* and saturation were observed in AL pigs, indicating a darker and redder meat [27]. This agrees with the previously mentioned higher myoglobin content in this genotype. Thus, when compared to BI, AL pigs showed a more intense red SM muscle, as observed in LL [14], which is a distinctive feature for the consumer [38]. The higher L^* values observed in SM samples from BI pigs could be partially associated to a higher muscle water loss in these pigs, already noticed in LL muscle [14]. In fact, the higher amount of muscle free-water provides a more reflective surface for light and is positively correlated to lightness [27,39]. Finally, colour values detected in muscle samples from RI crosses were overall intermediate to those observed in AL and BI genotypes, except for H°, closer to the AL values in BI × AL and to the BI values in AL × BI pigs. The lower H° values observed in BI × AL pigs measure a colour closer to the true red axis and agree with a significantly high content in myoglobin in these pigs. Similar results were previously observed in biceps femoris from Duroc × Iberian pigs, when compared to Iberian × Duroc [40].

DSF chemical composition was also affected by genotype, with AL pigs showing a 33.6% lower moisture and an 18.9% higher total lipids content than BI pigs, confirming precociously high adipogenic activity of AL [4].

4.2. Trial 2: Pigs Slaughtered at ~150 kg BW

Growth and carcass data from this trial were presented and discussed by Martins et al. [14]. Briefly, in the fattening period AL pigs had a lighter bone structure and a more compact body than BI, presenting a shorter carcass, and lower bone cuts proportions. Carcass yields, higher in these older and heavier pigs, increased 1.16, 0.80, 1.17, and 0.97 percentage units for each 10 kg increase in BW

from 65 to 150 kg in AL, BI, AL × BI, and BI × AL pigs, respectively. This confirms fat deposition as the main responsible for increasing carcass yield in older pigs [41,42]. AL pigs also showed a higher fat cuts proportion and a higher backfat thickness and ZP fat depth than BI, influencing the lean-to-fat cuts ratio, lower in AL pigs when compared to BI, and with RI crosses showing intermediate values.

SM muscle was only affected by genotype in total collagen content and pH_u parameters. IMF was 11% higher in AL than in BI pigs, however this difference did not attain statistical difference. When compared to BI, SM muscle samples from AL pigs also showed a higher percentage of IMF at 65 than at 150 kg BW (+23.4 and +11.1%, respectively). This was also observed in LL [14], suggesting that AL is an early maturing breed. Total protein values from AL were comparable to those previously reported for 100 kg castrated AL pigs fed at 85% ad libitum [20,28], but IMF values were higher, probably due to different feeding regimes and slaughter weight. Total protein and IMF values from BI pigs from our trial were identical to the ones reported by Carvalho [29].

Although the myoglobin content was 10.3% higher in SM from AL when compared to BI pigs, this difference did not attain statistical significance, contrary to what was observed in trial 1. Meanwhile, myoglobin content increased in all genotypes between 65 and 150 kg BW (186 and 354 days of age), showing that pork gained a more intense colour with age [27], as previously observed in Iberian pigs [43]. On the other hand, SM total collagen content observed in this trial in free-range AL pigs, was higher than the one in 100 kg confined AL pigs [20]. At 150 kg BW, SM total collagen and soluble collagen were also 25.1% lower and 20.6% higher in AL than in BI pigs, respectively. RI crosses also showed lower total collagen values than BI pigs. These differences suggest a higher tenderness of pork from AL pigs and RI crosses. Furthermore, ageing animals show a higher number of stable bonds between collagen molecules, with the corresponding decrease in its solubility [27,43,44], as observed in our pigs slaughtered at 65 and 150 kg. As animals age, meat becomes tougher, mainly due to an increase in the percentage of heat-insoluble collagen bonds [27]. This is a more important factor in local pig breeds than in industrial genotypes, because the former are slaughtered at physiologically older ages.

Ultimate pH (pH_u) of meat influences water-holding capacity, colour, tenderness, flavour and shelf life of meat [45] and therefore, is a main quality determinant [45,46]. The pH_u values observed in SM were within the normal range for pork [34], but were affected by genotype. As already observed in LL [14], pH_u values from SM samples were higher in AL than in BI pigs. This suggests a higher muscle glycogen content in BI pigs, positively correlated to lower pH values [47]. Generally, leaner animals have higher percentages of fast-contracting glycogen-rich type IIb or white fibres [48], with a glycolytic metabolism and higher ATP-ase activity, leading to lower pH_u values than those observed in slow-contracting oxidative type Ia or red fibres [46].

Meat colour, the major visual factor affecting meat quality [27], was influenced in a less expressive way by genotype in SM during fattening than during growth, as previously observed in LL [14]. Genotype only affected H°, which was significantly lower in SM samples from AL and AL × BI pigs. SM muscle from AL and AL × BI pigs also tended to show lower levels of L^* than BI pigs, but a^* values were not significantly different. A lower L^* and H°, as observed in AL and AL × BI pigs, is related to a darker and redder meat surface in terms of real colour perception [27]. Both darkness and redness can be enhanced at higher pH values, as observed in the SM muscle from AL pigs. In these conditions, reducing and oxygen-consuming enzymes are decreasing the percentage of myoglobin in the oxygenated form, and light scattering is minimized because hydrated muscle proteins are not releasing free water [39]. Overall, when comparing the two trials, an increase in age/weight led to a reduction of L^* and an increase of a^* values, generally associated to pork with a darker red colour [27]. This difference in L^* values from the SM muscle of AL and BI pigs was higher than two units in both trials, which could affect consumers preferences and influence the decision to purchase [49].

Chemical characteristics of GM muscle were slightly affected by genotype. IMF content was higher in AL and BI × AL pigs than in BI and AL × BI pigs, and was comparable to that previously observed in 103 kg BW Iberian pigs fed at 90% ad libitum [50]. Although the difference in IMF between AL and

BI was expected, due to the higher adipogenic activity of the former breed [4], IMF values observed in BI × AL, are interesting. In fact, when analysing the IMF values obtained in this trial, BI × AL pigs showed a fat content in both muscles numerically close to the one from AL pigs, suggesting a maternal effect. Since the technological quality of fresh meat and meat products is mainly determined by the lipid fraction, this higher IMF content in the two valuable ham muscles from BI × AL pigs, is very important. Similar results were obtained when comparing IMF content of biceps femoris from hams of Iberian pigs to those of Duroc boars × Iberian dams and of Iberian boars × Duroc dams [51]. Finally, GM total collagen observed in AL pigs and RI crosses was lower than the one in BI pigs. When associated to higher IMF values, as also observed in SM muscle (and in LL muscle—[14]), this suggests a higher tenderness of pork from AL pigs and RI crosses.

DSF chemical composition also varied among genotypes, with AL pigs showing a 29.5% lower total protein and a 6.2% higher total lipids content than BI. Such changes agree with the more adipogenic profile of AL when compared to the leaner BI pig [4,5]. However, histological studies are needed to clarify if the difference in protein content is related to collagen or fat deposition. The latter could be obtained either through an increased adipose cell number in BI pigs and/or by a cell hypertrophy in AL pigs (fewer cells per gram of subcutaneous tissue). When calculating the fat weight deposited in DSF ((DSF weight × % DSF lipids)/100) at 65 and 150 kg BW, the values were 2.88 and 6.31 kg, respectively. Once again, BI × AL pigs showed a total lipids content similar to that of AL, and higher than the one from BI and AL × BI pigs, which is important from a technological point of view. Finally, regarding DSF colour, AL pigs had a 33% lower a^* value than BI, which affected C*, H°, and saturation values. The higher lipid concentration in DSF of AL pigs may have contributed to the dilution of blood vessels in this tissue, leading to lower values of a^* and saturation, also observed in 65 kg pigs but without attaining statistical significance. In fact, haemoglobin, the major colour pigment in blood, can also affect tissue colour [27].

5. Conclusions

Data obtained at the growing period showed that Alentejano (AL) is a fatty breed, with lower lean and higher fat cut proportions than Bísaro (BI) [14]. SM muscle from AL pigs showed higher IMF, redder colour, and lower total collagen, features that could positively influence the consumer from a visual and/or an eating quality point of view. Ribatejano (RI) reciprocal crosses (AL × BI and BI × AL) showed overall intermediate features between AL and BI genotypes, but higher lean and lower fat cut proportions and backfat thickness than AL. On the other hand, they showed a SM muscle with a myoglobin content and colour characteristics in line with those observed in AL pigs. This suggests a redder and darker meat than the one from BI pigs, at a slaughter weight generally used for meat production. These features were overall similar in both muscles of pigs slaughtered at the end of the fattening period (~150 kg BW). At this slaughter weight, muscles from RI crosses also had a lower total collagen content, suggesting a darker, redder, and more tender meat for fresh and cured products than the one from BI pigs. Therefore, RI crosses have the potential to be sustainably reared outdoors and to produce high quality meat and fermented or dry-cured products. AL × BI, more easily reared in the north of Portugal (BI dams' homeland), could improve the meat and meat products quality when compared to the ones obtained with pure BI pigs. As to BI × AL, more easily reared in the south (AL dams' homeland), this cross has better commercial yield and primal cuts proportions, when compared to those obtained from pure AL pigs, without compromising meat and meat products quality. Finally, the production of high quality/certified products to attain better market prices can lead producers to increase animal and productivity numbers and therefore contribute to maintaining or increasing animal biodiversity.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/10/5/905/s1, Table S1. Chemical composition (g/100 g) of the commercial diets fed to Alentejano (AL), Bísaro (BI), AL × BI and BI × AL pigs slaughtered at ~65 and 150 kg BW.

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Chapter 4

Comparative Transcriptomic Analysis of Subcutaneous Adipose Tissue from Local Pig Breeds – article #3

Albuquerque, A.; Óvilo, C.; Núñez, Y.; Benítez, R.; López-Garcia, A.; García, F.; Félix, M.d.R.; Laranjo, M.; Charneca, R.; Martins, J.M. Comparative Transcriptomic Analysis of Subcutaneous Adipose Tissue from Local Pig Breeds. 2020. *Genes*, *11*, 422. https://doi.org/10.3390/genes11040422

Supplementary materials are available online: http://www.mdpi.com/2073-4425/11/4/422/s1

Note: The complete list of differentially expressed genes can be found in Appendix II.

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Comparative Transcriptomic Analysis of Subcutaneous Adipose Tissue from Local Pig Breeds

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Abstract: When compared to modern lean-type breeds, Portuguese local Alentejano (AL) and Bísaro (BI) pig breeds present a high potential for subcutaneous and intramuscular fat (IMF) deposition which contributes for better meat quality. The aim of this work was to explore the genome function to better understand the underlying physiological mechanisms associated with body fat accretion. Dorsal subcutaneous fat samples were collected at slaughter from adult animals ($n = 4$ for each breed) with ~150 kg body weight. Total RNA was obtained and sequenced for transcriptome analysis using DESeq2. A total of 458 differentially expressed (DE) genes (q-value < 0.05) were identified, with 263 overexpressed in AL and 195 in BI. Key genes involved in *de novo* fatty acid biosynthesis, elongation and desaturation were upregulated in AL such as *ACLY*, *FASN*, *ME1*, *ELOVL6* and *SCD*. A functional enrichment analysis of the DE genes was performed using Ingenuity Pathway Analysis. Cholesterol synthesis is suggested to be higher in AL via SREBF2, SCAP and PPARG, while lipolytic activity may be more active in BI through GH and AMPK signalling. Increased signalling of CD40 together with the predicted activation of INSIG1 and INSIG2 in BI suggests that this breed is more sensitive to insulin whereas the AL is less sensitive like the Iberian breed.

Keywords: Alentejano pig; Bísaro pig; RNA-seq; differentially expressed genes (DEGs); Dorsal subcutaneous fat; transcriptome

1. Introduction

Pork meat represents one of the main sources of protein, fat and iron for humans, accounting for about 30% of meat consumption worldwide. Fresh meat market is essentially dominated by a few genetically selected breeds with notable productive traits and raised under intensive conditions [1]. However, in the last decades, a growing interest for better meat quality has increased the tendency for native breeds to prosper [2,3]. These breeds are generally well adapted to the local environment and subsist in small populations with diversified and accessible food from natural resources. Alongside their

inherent genetic value for biodiversity, local breeds are used to produce high quality dry-cured meat products, representing an important role in local economies, culture and landscape as the basics of sustainable local pork chains [2,4].

Alentejano (AL) and Bísaro (BI) are the two main Portuguese pig breeds. AL evolved from the primitive *Sus scrofa mediterraneus*, belongs to the Mediterranean group of breeds [5] and is genetically similar to the Iberian (IB) pig [6]. This breed is commonly raised in the south of Portugal and generally characterized by its light bone structure, black color, short and slim extremities and energetic nature [7]. A medium-sized pig, the AL grows at a low rate (except under the finishing phase "montanheira") and presents a low prolificacy [8]. On the other hand, its high and early adipogenic activity provides a meat and fat composition that is attractive for both fresh meat market and for processing high-grade sausage and dry cured products [9,10]. Traditionally, the AL pig is raised under extensive conditions in an integrated pastoral system ("montado") and during the fattening season is fed with acorns from the existing Quercus forests from October to February [5,7]. BI breed belongs to the Celtic group [5], sharing ancestors with highly productive breeds such as Large-White and Landrace. Production of BI is distributed throughout the north of Portugal, from the Tagus River to the northern border with Spain. It is characterized by its docile temperament, presenting a large body with large legs, head and shoulders. BI pig presents a higher prolificacy and productivity than AL, though lower than other genotypes with similar origins but raised under intensive production systems and subjected to genetic improvement programs [11–14]. On the other hand, the BI pig presents a lower adipogenic trend when compared to AL, but still higher than most modern lean breeds, which leads to a medium fat carcass with an overall good sensorial quality of meat and capability to further process into high-grade meat products [15,16]. Traditionally, BI production is based on small-scale family farms with a low number of sows, fed with domestic food scraps and horticultural by-products [17,18]. Nowadays, the number of sows per farm has increased, while reared by smallholders and in medium-sized farms based on free-ranged systems, most of them allowing access to pasture [19].

The old perception of adipocytes as a mere energy storage tissue is nowadays incomplete due to its extensive autocrine, paracrine and endocrine activities via liberation of various specific cytokines that modulate gene expression and nutrient flow to balance current metabolic needs [20]. Identification of these mechanisms and how they interact can help understand and treat associated metabolic diseases such as obesity and type-2 diabetes. Meanwhile, adipocyte size and number set the overall fatness attribute of a carcass that is essential for the marbling trait preferred by consumers which is described by the presence of an acceptable amount of intramuscular fat (IMF) [21]. Subcutaneous fat, on the other hand, is found beneath the skin in several layers separated by connective tissue, with the breeds overall potential for fat deposition affecting the full development of the most inner layer [21].

Lipid accumulation and further deposition precede a shift in the metabolic balance, favoring lipogenesis and adipogenesis over catabolic pathways such as β-oxidation and is consequence of an excessive caloric intake. The AL, as the Iberian pig and other Mediterranean breeds, feature a genetic predisposition to gain and preserve subcutaneous and IMF, referred as the thrifty genotype [22]. This susceptibility was historically advantageous when animals had to endure seasonal periods of starvation and the excess fat stores developed during periods of abundance were valuable sources of energy for survival [23]. Fatter animals generally present increased levels of circulating leptin and insulin. Leptin is secreted by the adipocytes as an attempt to control appetite and food intake [24], however humans and animals naturally propense to become obese, such as the IB pig, are found to display a pattern of resistance to leptin [25]. In IB pigs, leptin-resistance is at least partially justified by the presence of a fixed functional polymorphism in the leptin receptor gene [26,27] which was recently found as almost fixed in the AL (0.98) and at a much lower frequency in the BI breed (0.26) [6]. Obese animals also secrete more insulin in order to control circulating glucose levels by inducing glucose uptake and storage as glycogen, while triggering lipogenesis and preadipocyte differentiation [28,29]. On the other hand, insulin sensitivity has been previously proposed as a major factor to justify differences in phenotypical traits such as growth and body composition between local

and lean modern breeds [30]. A recent study did, in fact, determine that growing IB pigs can develop insulin resistance at an early stage and that insulin is less effective in IB than in Landrace pigs [30]. A similar pattern of diminished insulin sensitivity and possible insulin resistance is therefore possible to develop in AL, particularly when compared to the leaner BI pig. Insulin resistance is characterized by an over secretion of insulin resulting in hyperinsulinemia that induces triglyceride accumulation and lipolysis inhibition in tissues via glucose transporter-4 (GLUT4), mediated by the phosphatidylinositol 3-kinase (PI3K) signaling pathway [29,31]. Furthermore, obese insulin resistant animals secrete more proinflammatory cytokines, which in turn contribute to the development of an obesity-induced chronic inflammation state [32]. These attributes show the potential and justify the continuing application of local pig breeds as biomedical models to study worldwide growing metabolic related disorders such as obesity, type-2 diabetes mellitus and cardiovascular diseases [25].

Genetic potential, along with specific feeding and production strategies, plays a major role on the development and composition of tissues. This work was intended to explore the genome function of AL and BI pig breeds at the level of adipose tissue, to better understand the underlying mechanisms associated with lipid deposition and productive traits of these breeds. This RNA-seq comparative analysis represents the first high-throughput transcriptomic data involving these local breeds and can help explain the metabolic differences that occur in their adipose tissue.

2. Materials and Methods

2.1. Animals, Experimental Design and Sampling

Purebred male castrated AL and BI pigs (n = 4 for each breed) were reared in a traditional free ranged system and individually fed commercial diets ad libitum until slaughter (~150 kg), under identical conditions to minimize non-genetic effects. AL pigs averaged a total of 155.0 days on trial while BI pigs averaged a total of 140.0 days on trial. Dorsal subcutaneous fat samples (DSF) were collected at slaughter as previously described [33], snap frozen in liquid nitrogen and maintained at −80 °C until total RNA extraction.

All animals were raised and slaughtered in conformity with the regulations and ethical guidelines of the Portuguese Animal Nutrition and Welfare Commission (DGAV, Lisbon, Portugal) following the 2010/63/EU Directive. Staff members of the team involved in animal trials were certified for conducting live animal experiments delivered by the Directorate of Animal Protection (DSPA, DGAV, Lisbon, Portugal).

2.2. RNA Extraction and Sequencing

Total RNA was isolated from 50–100 mg samples of DSF following Ambion® RiboPure™ Kit (Thermo Fisher Scientific, Waltham, MA, USA) instructions. Total extracted quantity was measured using NanoDropTM 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Quality control was assessed using Agilent 2100 BioanalyzerTM (Agilent Technologies, Santa Clara, CA, USA) following Agilent RNA 6000 Nano Kit instructions, along with NanoDropTM 1000 260/280 and 260/230 coefficients that were checked for protein and chemical contamination, respectively. RIN values ranged from 6.5~8.5. The obtained total RNA was diluted into a concentration of 100 ng/μL and ~3 μg samples were sent for stranded paired-end mRNA-seq sequencing in Centro Nacional de Análisis Genómico (CNAG-CRG, Barcelona, Spain) on a HiSeq2000 sequence analyzer (Ilumina, Inc., San Diego, CA, USA). The raw data was downloaded from CNAG servers and treated accordingly.

2.3. Quality Control, Mapping and Assembly

FastQC program (version 0.11.8) [34] was run to assess the quality of the sequencing Fastq files. Sequence reads were trimmed three consecutive times for Ilumina adapters, poli-A and poli-T tails using Trim Galore (version 0.5.0) [35] while removing resulting low quality nucleotides (Phred Score, Q < 20) and short length reads (<40). The remaining reads were aligned to the reference pig genome

version Sscrofa11.1 (Ensembl release 94) using HISAT2 version 2.1.0. Resulting SAM files were then converted to BAM with Samtools-1.9 [36] and HTSeq-count version 0.11.1 [37] was used to count and merge reads based on overlapping paired-end reads.

2.4. Differential Expression Analysis

Previously generated Gcount files were run with the R package DESeq2 [38]. In this tool, gene expression levels are obtained through count of total exon reads for the statistical analysis. DESeq2 sets up normalized counts that were filtered by the rule of minimum 50 reads per group. Genes were considered as DE when presenting a false discovery rate (FDR) or adjusted *p*-value lower than 0.05. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [39] and are accessible through GEO Series accession number GSE145956 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145956).

2.5. Functional Enrichment Analysis

To explore causal relationships associated with the resulting DE genes, predict downstream effects, identify new targets and better interpret their biological meaning within the adipose tissue context, a functional enrichment analysis was performed using Ingenuity Pathways Analysis software (IPA; QIAGEN). The list of candidate DE genes ($q < 0.05$) and their respective log2 FC ratio was uploaded into the application and then converged with IPA's library (Ingenuity Pathway Knowledge Base) to identify biologically relevant information such as overrepresented pathways and functions, networks and regulators [40].

2.6. Real Time Quantitative PCR and Statistical Analysis

Previously extracted total RNA was reverse transcribed in 20 µL reactions using Maxima® First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, MA, USA) following manufacturer's instructions. In order to validate the data generated by RNA sequencing, Real Time-qPCR was performed for a set of 9 candidate genes including *ACACA*, *ACLY*, *ADIPOQ*, *ELOVL6*, *FASN*, *LEP*, *ME1*, *PCK1* and *SCD* (Supplementary Table S1). Standard PCRs were executed to check amplicon sizes.

Quantification reactions containing 12.5 µL of NZY qPCR Green Master Mix (2×) (NZYtech, Lisbon, Portugal), 0.3 µM of each respective sense primer and 12.5 ng of cDNA per sample were prepared in 96-well plates and run in a LineGene9600 Plus system (BIOER, Hangzhou, China). PCR program comprised an initial hold 10 min denaturation stage at 95 °C, followed by a 40 cycles amplification step of 15 s denaturation at 95 °C and 50 s at the respective primer pair annealing temperature (Supplementary Table S1). A melting stage to test PCR specificity was also added at the end involving a single cycle at 95 °C (15 s) followed by 60 °C (60 s), and a ramp-up 0.2 °C/s to 95 °C for 15 s with acquired fluorescence. Single peaks in the dissociation curves confirmed the specific amplification of the genes. A no-template control was run with every assay, and target samples were performed in triplicate as technical replicates. Ct values were regressed on the log of template cDNA concentration. For each gene, PCR efficiency was estimated by standard curve calculation [41] using five points of cDNA serial dilutions (1:4; 1:8; 1:16; 1:32; 1:64). *ACTB*, *RPL19* and *TOP2B* were selected as endogenous genes for normalization of target genes and their stability was assessed using the Genorm software ($0.271 < M < 0.363$) [42].

An Independent Samples t-test was performed on the dataset using IBM SPSS Statistics software (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp) with significance defined as $p < 0.05$. Equal variances were tested with Levene's Test for Equality of Variances. Significance levels lower than 0.05 were not considered as equal variances and another Independent Samples Test was performed assuming no equal variances. Equal variances were not assumed for *ACLY* ($F = 12.5$; $p = 0.01$) and *ME1* ($F = 32.3$; $p = 0.001$). Pearson correlation coefficients and associated *p*-values were estimated.

To measure the level of agreement between RNA-seq and Real time qPCR results, the concordance correlation coefficient (CCC) [43] was estimated using the Log2FC values per candidate gene.

3. Results and Discussion

3.1. Alentejano and Bísaro: Local Pig Breeds with Recognizable Meat Quality Traits

The animals tested in this study, purebred AL and BI pigs differ phenotypically, genetically and regarding their respective traditional production systems. These breeds represent the two most important local pig breeds produced in Portugal, and there is a renewed interest in their resulting crosses, the Ribatejano (RI) pig [11]. In a previous study [33], a comparison of productive and meat quality traits was analysed in the AL, BI and RI pigs, with BI presenting significantly better carcass traits than AL and intermediate values for the crossed pigs. From this study, four randomly selected individuals from AL and BI breeds were chosen for transcriptome analysis by RNA-seq. Currently, many RNA-seq experiments are performed at a low replication level and several publications suggest that a minimum of 2–3 replicates can be considered [44–46]. Selected AL pigs averaged a total of 155.0 days on trial with an average daily gain of 571.6 g/d while BI pigs averaged a total of 140.0 days on trial and an average daily gain of 619.4 g/d. On the other hand, when compared to BI, AL pigs presented significantly higher fat cuts proportions (32.0 vs. 25.4%, $p < 0.05$), average backfat thickness (78.6 vs. 45.1 mm, $p < 0.01$) and fat depth (69.6 vs. 36.5 mm, $p < 0.01$). BI pigs on the other hand presented higher primal cuts proportions (33.2 vs. 26.1%, $p < 0.01$), bone cuts proportion (13.8 vs. 11.8%, $p < 0.05$) and lean-to-fat cuts ratio (1.89 vs. 1.41, $p < 0.05$).

3.2. Mapping and Annotation

The average number of obtained sequenced reads was 45 million per sample. Length of reads was consistently 76 bp with the average associated quality score roughly close to 40. GC content ranged from 49 to 56%. Resulting trimmed paired-end reads were mapped to the reference genome Sscrofa11.1 with a consistent alignment rate of 97% using HISAT2 over all samples, which is higher than most previous pig transcriptome studies [47–52] and is probably due to an improved annotated reference genome.

3.3. Gene Expression Analysis: DESeq2

Previously obtained normalized counts were used to predict and establish the total number of DEG's between breeds. Over 10.7 K genes were detected across all samples with 458 found to have significantly different expression values between breeds ($q < 0.05$). A total of 263 genes were found overexpressed in AL and 195 in BI with 47 (20%) of total DEG's still labelled as novel genes with hardly any information available on most databases. A full detailed list of the total DEG's can be found in Supplementary Table S2. The novel gene coding for the baculoviral inhibitor of apoptosis protein repeat containing 7 (*BIRC7*) was the most overexpressed in the AL breed (log2 FC = 4.86, $q < 0.01$) while Taste receptor type 2 member 39 (*TAS2R39*) was the most overexpressed in BI (log2 FC = −4.61, $q < 0.05$). In humans, *TAS2R39* plays a role in the perception of bitterness and is linked with the G protein associated with taste and the gustatory system [53]. In pigs, taste perception can influence feeding patterns which can consequently determine production traits and *TAS2R39* in particular has been previously associated with increased lipid deposition [54]. A recent study that investigated the allele frequency of known polymorphisms with associated meat quality traits in European local pig breeds [6], including AL and BI, found that the missense mutation p.Leu37Ser in *TAS2R39* associated with higher backfat deposition is practically absent in AL (0.01), while in BI is present in 13% of individuals. This contradicts phenotypic data and suggests that regulation of lipid content may occur at different levels and differ in pathways from AL pigs.

Overall, and as expected, genes encoding for enzymes or transcription factors involved in lipid synthesis were found overexpressed in AL. Furthermore, we confirmed that, in this breed, several

major genes involved in the cascade of the de novo lipid synthesis, elongation for very long fatty acids and desaturation, were upregulated (Figure 1).

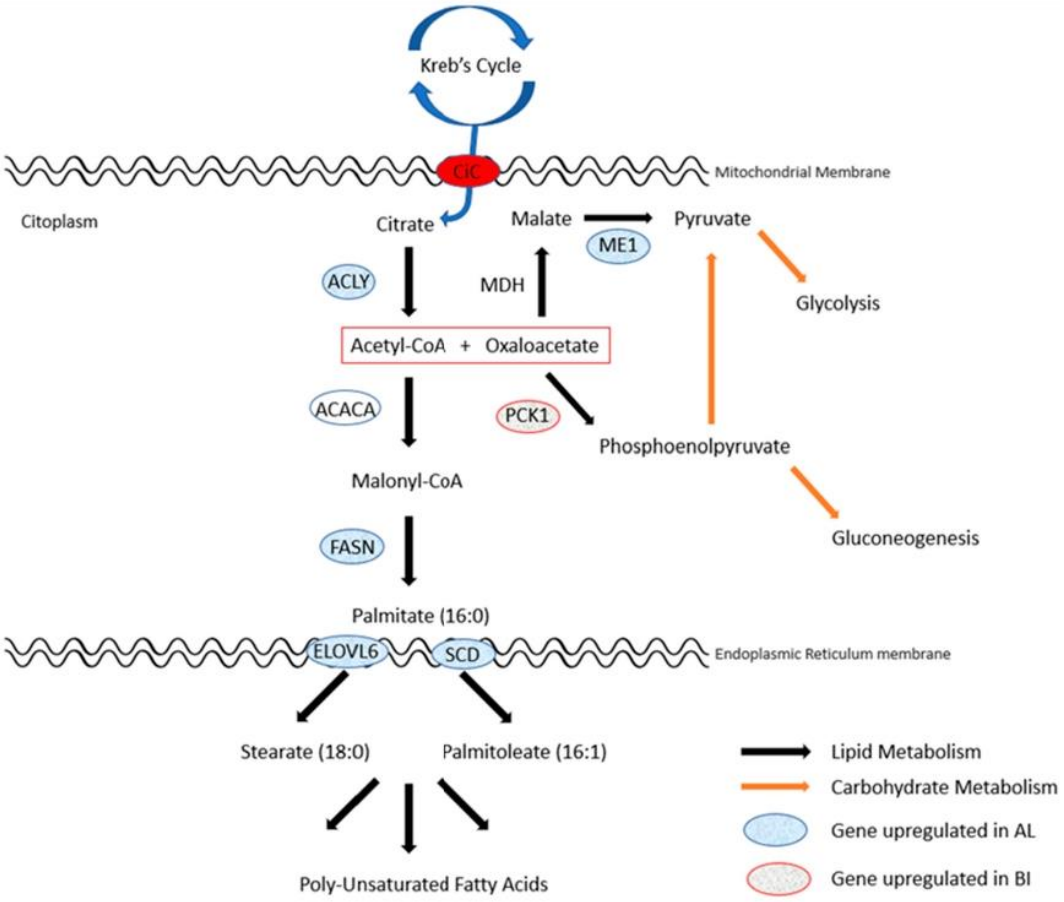

Figure 1. Linking lipid biosynthesis with carbohydrate metabolism: direction of upregulated genes in the Portuguese local pig breeds.

ATP citrate lyase (*ACLY*) was found significantly overexpressed in AL when compared to BI (log2 FC = 1.853, q < 0.01). ACLY is the primary enzyme responsible for the synthesis of cytosolic acetyl-CoA in many tissues. It catalyses the reaction where the citrate produced through Krebs Cycle, freely transported to the cytoplasm by the Citrate Carrier (CiC), is converted into acetyl-CoA and oxaloacetate [55,56], along with the hydrolysis of ATP, linking the carbohydrate to the lipid metabolisms. Acetyl-CoA is the main non-lipid precursor, with NADPH, for the synthesis of cholesterol and/or palmitic acid through *de novo* synthesis [57].

The first step to synthesize new fatty acids comprises the carboxylation of acetyl-CoA into malonyl-CoA [57], a reaction catalysed by the enzyme acetyl CoA carboxylase (ACACA) who's gene was found in our data to be non-statistically different between breeds though a numerical difference was noted (log2 FC = 0.854, q = 0.116). That difference was then confirmed and boosted to trend by qPCR (log2 FC = 1.055, $p = 0.077$).

Long carbon chain FAs are then assembled in multiple repeated four-step cycles comprehending: condensation, reduction, dehydration, and reduction. In each cycle two carbon atoms are added to the acyl chain, which is the substrate for the next cycle with an activated malonyl CoA group [58]. For the complete synthesis of palmitic acid (16:0) the cycle must be repeated seven consecutive times, a reaction operated by the multifunctional enzyme complex fatty acid synthase (FASN) that requires

much NADPH. In our data, *FASN* was overexpressed in AL (log2 FC = 1.691, q < 0.01) validating the concept of an increased lipid biosynthesis in AL when compared to BI.

Elongation of saturated and/or unsaturated FAs occurs in the cytoplasmic face of the endoplasmic reticulum membrane where the elongation of very long chain fatty acids protein 6 (ELOVL6) plays a pivotal role by adding two-carbon atoms from a malonyl CoA donor [59]. *ELOVL6* was found also significantly overexpressed in AL (Log2 FC = 1.236; q < 0.01) suggesting a higher production of long chain FAs, particularly stearic (18:0) and oleic (18:1) acids.

The acyl CoA desaturation also occurs at the endoplasmic reticulum. Stearoyl CoA desaturase (SCD), together with NADH-cytochrome b_5 reductase and cytochrome b_5, work as a membrane-bound complex to catalyse the reaction that introduces a double bond between C9 and C10 [60] to mainly create palmitoleic (16:1) and/or oleic acid which are major components of membrane phospholipids, triacylglycerols and cholesterol esters [58,60]. Furthermore, oleic acid is the starting point to assemble a variety of unsaturated FAs by a combination of elongation and desaturation reactions. SCD is the rate-limiting step of the reaction and, in our data, *SCD* gene was also found significantly overexpressed in AL (log2 FC = 2.311, q < 0.026). This suggests a higher synthesis of oleic acid and its derivatives in this genotype, which lead to a higher oleic acid content in meat as found in previous studies [7,9,61].

When citrate is converted to acetyl-CoA and oxaloacetate, the later can be converted to malate through the action of malate dehydrogenase (MDH) and then to pyruvate to enter the glycolytic pathway [62]. The latter reaction is catalysed by malic enzyme 1 (ME1) and is characterized by the reversible oxidative decarboxylation of malate generating much of the NADPH supply required for lipid biosynthesis [63]. In our data, *ME1* gene was also found significantly overexpressed in AL (log2 FC = 2.090, q < 0.01) supporting the idea of an overall increased lipid synthesis in this breed.

Phosphoenolpyruvate carboxykinase 1 (PCK1) is responsible for catalysing the reaction where cytosolic oxaloacetate is converted to the intermediate phosphoenolpyruvate which can participate in gluconeogenesis or glycolysis when converted to pyruvate [57]. Moreover, PCK1 has been found to regulate free fatty acid reesterification and glyceroneogenesis in white adipose tissue [64]. Contrarily to what was found in another study using Iberian when compared to Duroc pigs [52], *PCK1* gene was found significantly overexpressed in BI (log2 FC = −1.959, q < 0.05) which suggest that in BI the path of gluconeogenesis/glyceroneogenesis pathway is favoured. A single nucleotide polymorphism c.A2456C in the *PCK1* gene has been reported to favour higher intramuscular deposition and better meat quality in pigs muscle [65]. The presence of this mutation in AL and BI genotypes was recently studied [51] showing that AL presents the A allele practically fixed (0.97) as do the IB (0.96). However, the C allele is present in BI in approximately 50% of the animals. The C allele causes the integration of leucine instead of methionine at the position 139 of the enzyme, which is associated with higher meat water loss and less favourable fat distribution. PCK1 p.139Met is kinetically more active in the direction of phosphoenolpyruvate synthesis for glyceroneogenesis while the PCK1 p.139Leu (allele A) is more active in the opposite direction, leading to oxaloacetate synthesis [65] which may contribute to the less fatty phenotype of the BI breed contrasting to the higher lipid deposition and lower carcass traits.

Leptin (LEP) is a homeostatic hormone mediator mainly expressed in adipocytes. It is widely recognized for regulating food intake and energy expenditure at the hypothalamic level [66] although the exact working mechanism in the adipose tissue continue unclear. Circulating levels of LEP are consistently higher in fatty animals. LEP targets peripheral tissues favouring FA catabolism over lipogenesis and it has been suggested that leptin can regulate adipose tissue metabolism by autocrine signalling [67]. As in other studies with the Iberian pig breed [52,68,69], genetically similar to AL, compared to the leaner Duroc and Landrace breeds, *LEP* gene was found significantly overexpressed in the AL pig (log2 FC = 1.376, q < 0.05). This is in agreement with its recognized fatter phenotype and, very similar to the IB breed, suggesting the development of leptin resistance [27,52].

The CCAAT/enhancer-binding protein α (*CEBPA*) gene encodes a transcription factor, containing a basic-leucine zipper domain that recognizes the CCAAT sequence in the promoter region of specific target genes that indirectly participates in the regulation of multiple pathways, including glucose and

lipid metabolism [70,71]. In humans, several studies have found a lower expression of *CEBPA* in obese groups [72,73] despite being usually associated to adipogenesis, adipose tissue development and lipid accumulation [71,74]. However, in our study, *CEBPA* was found upregulated in the obese AL (log2 FC = 0.974, $q < 0.05$) supporting the concept of increased lipid synthesis in this breed.

The fatty acid binding protein 4 (*FABP4*) gene encodes for a cytoplasmic protein found in adipocytes that binds long-chain fatty acids and plays an essential role in lipid metabolism and homeostasis. In cattle, FABP4 has been associated with better marbling and fat depth [75] and in pigs is a recognized genetic marker for meat tenderness and IMF content [76]. FABP4 is also known to indirectly influence adipogenesis and insulin responsiveness by adjusting the master regulator peroxisome proliferator-activated receptor gamma (PPARG) [77]. In our data, *FABP4* was found significantly overexpressed in AL (log2 FC = 1.061, $q < 0.01$), which agrees with the frequently higher expression values found in obese individuals [77]. Higher FABP4 levels are induced by increased levels of insulin or insulin-like growth factor-1 (IGF1) and are frequently associated with the development of insulin resistance [78].

Adipocyte expression of IGF2 has been previously associated with enhanced foetal growth and subcutaneous preadipocyte differentiation via PPARG activation while decreasing fat deposition in visceral preadipocytes [79]. In pigs, *IGF2* is a candidate gene for meat production and carcass traits [80]. An intronic mutation in *IGF2* (g.3072G > A) is strongly related to fat depot and muscle development when the G allele is present [81]. In AL pigs the G allele is fixed while in BI the A allele is residual (0.01) [6]. *IGF2* was found significantly overexpressed in BI (log2 FC = −0.884, $p < 0.05$) despite their leaner phenotype which indicates that fat deposition is regulated at other levels. This result is in agreement with previous studies that found IGF2 overexpression in leaner Duroc when compared to the fatter Iberian pig genotype [52].

Similar to what was found in a recent study [52] that compared the transcriptome of animals with distinct tissue distribution (Iberian vs. Duroc), a set of genes that play an important role in growth and development was, within our dataset, consistently found overexpressed in BI, the breed with the highest lean muscle tissue deposition. Those genes included *IGF2* (log2 FC = −0.884, $q < 0.05$), *FOS* (log2 FC = −1.087, $q < 0.05$) and *FOSB* (log2 FC = −2.028, $q < 0.01$). Moreover, several other genes associated with proliferation of muscle cells and cellular development were consistently found overexpressed in this local breed, including *APOD* (log2 FC = −1.087, $q < 0.05$), *DUSP1* (log2 FC = −1.539, $q < 0.01$), *EGR1* (log2 FC = −1.172, $q < 0.05$), *ELN* (log2 FC = −1.743, $q < 0.01$), *KLF4* (log2 FC = −1.103, $q < 0.05$) and *STAT3* (log2 FC = −0.940, $q < 0.05$). These findings agree with other previous works [49,52,82] and support the idea that growth and development stimuli, particularly in the more productive breeds, can occur and be regulated at a multiple tissue level.

3.4. Validation by Real Time qPCR

In order to validate the RNA-seq results, the relative expression of a selected group of DE genes as well as some non-DE genes was assessed by semi-quantitative Real Time PCR within the same sampling universe (Table 1). Overall, RNA-seq results agreed with the results obtained from real time qPCR, despite occasional small significance inconsistencies within some genes probably due to particularities of each method regarding accuracy, sensitivity and specificity.

As found in other studies [49], statistically significant differences generally tend to be higher with the RNA-seq approach when compared to the Real-Time qPCR technology where *p*-values are higher. A concordance correlation coefficient (CCC), usually employed to measure the degree of agreement between two variables in order to evaluate the reproducibility of tested methods, was determined between the RNA-seq and qPCR methods. The obtained value of 0.804 demonstrates a substantial strength-of-agreement between the treatments.

Table 1. Gene expression comparison through RNA-seq and Real Time qPCR of the selected genes.

Genes	RNA-seq		Real Time qPCR		Correlation	
	Log2 FC	q-Value	Log2 FC	*p*-Value	*r*	*p*-Value
ACACA	0.854	0.116	1.055	0.077	0.986	7.07×10^{-6}
ACLY	1.853	0.005	1.601	0.068	0.864	0.006
ADIPOQ	−0.625	0.297	−0.685	0.110	0.707	0.050
ELOVL6	1.236	0.009	0.671	0.136	0.650	0.081
FASN	1.691	0.002	1.359	0.100	0.920	0.001
LEP	1.376	0.037	0.929	0.046	0.739	0.036
ME1	2.090	8.42×10^{-6}	1.008	0.106	0.757	0.030
PCK1	−1.959	0.037	−2.660	0.001	0.820	0.013
SCD	2.311	0.026	1.351	0.087	0.643	0.086
			$n = 4$			

3.5. Functional Analysis

IPA software was used to find overrepresented biological functions, pathways and potential upstream regulators within the candidate DEG dataset, where 380 of the total 458 candidate DEG's were recognized by its database.

Gene Ontology enrichment analysis revealed a total of 500 involved biological functions ($p < 0.05$) within our DEG dataset (Supplementary Table S3), with 17 of these being significantly activated (z-score ≤ −2 or ≥2) in a breed. A total of 5 functions related either to development and growth or cell death were predicted to be activated in BI (z-score ≤ −2) including generation of embryonic cell lines, necrosis and cell hypoplasia. On the other hand, 12 biological functions mostly related to cell-signalling and cellular development were predicted to be activated in AL (z-score ≥ 2), including cell proliferation of hepatoma cell lines, activation of myeloid cells, proliferation of fibroblast cell lines and activation of granulocytes. Other biological functions of interest were found significantly associated to our gene dataset, but no directional prediction was attained. Among them, size of animal, gluconeogenesis, quantity of glycogen, synthesis of lipid, concentration of fatty acids, quantity of white adipose tissue, concentration of triacylglycerols, synthesis of sterols, proliferation of muscle cells, inflammatory response and insulin sensitivity. As reported in a similar study that compared the breed effect on the adipose tissue transcriptome [52], our results suggest that a highly activated inflammatory state, frequently defined as meta-inflammation, may have developed in the adipose tissue due to the large fat mass attained, particularly in AL pigs.

A total of 57 canonical pathways were found associated with our DEG dataset ($p < 0.05$) (Supplementary Table S4). Two of them were found within the scope of a directional prediction (z-score ≤ −2; z-score ≥ 2), namely CD40 signalling (z-score = −2.236) and mouse embryonic stem cell pluripotency (z-score = −2.236). *CD40* encodes for a protein member of the TNF-receptor superfamily which operates as a receptor for immune system cells and participate in the regulation of numerous immune and inflammatory responses [83]. Studies in mice [84,85], have pointed the regulatory role of CD40 in obesity-induced insulin resistance and has associated CD40 deficiency with increased inflammation and decreased insulin sensitivity, pointing its potential in preventing obesity and metabolic disorders. The fact that CD40 signalling is one main canonical pathways found and that is significantly favoured in BI, supports the idea that insulin resistance is mainly occurring in the AL breed, where decreased insulin sensitivity is suggested due to the pro-inflammatory effect of the exacerbated level of adipose tissue deposition when compared to the leaner BI.

The top canonical pathway detected was Growth Hormone (GH) Signalling ($p = 1.95 \times 10^{-4}$), followed by the HOTAIR regulatory pathway ($p = 3.80 \times 10^{-4}$), both suggested to be activated in BI (−0.378 and −0.632 of z-score, respectively). GH is widely recognized for its anabolic and lipolytic functions, stimulating muscle and bone growth mediated by IGF1 secretion. In adipocytes, stimulated GH receptors induce lipolysis through oxidation and triacylglycerol breakdown [86]. On the other

hand, the homeobox transcript antisense intergenic RNA (HOTAIR) is part of the long noncoding RNAs class that is suggested to regulate the expression of numerous signalling transcripts and is a potential biomarker for several types of cancer [87]. Recent studies have also associated upregulated levels of HOTAIR with increased adipogenesis, through upregulation of *FASN* [88,89], which suggest a different regulatory pathway in BI compared to the AL breed, that presented a higher expression of *FASN*.

Other interesting canonical pathways associated to BI include prolactin (z-score = −0.447, $p = 0.002$) and signalling of a few specific regulators such as AMP-activated protein kinase (AMPK) (z-score = −1.134, $p = 0.01$), IGF1 (z-score = −0.447, $p = 0.048$) and cytokines interleukin-2 (IL-2) (z-score = −1.000, $p = 0.019$) and IL-3 (z-score = −1.000, $p = 0.019$). These pathways suggest a higher lipolytic activity within the BI adipocytes, along with the activation of anti-inflammatory signals that may help control insulin sensitivity. Prolactin plays a major role in lactation and can also regulate the reproductive and immune systems. Metabolically, prolactin has been associated with a reduced ability for glucose and lipid deposition in human adipocytes, leading to higher circulating values of these compounds [90]. Patients with hyperprolactinemia are found with metabolic disorders such as insulin resistance and glucose intolerance [91]. Moreover, prolactin and GH can affect the expression of adiponectin, the adipose tissue regulator of glucose and fatty acid levels [92]. However, and as mentioned before, *ADIPOQ* expression values were found numerically higher (q = 0.297) in BI with RNA-seq as well as with real-time PCR ($p = 0.11$). AMPK has a key role in the cellular maintenance of ATP levels and is responsible for the regulation of growth and transcriptional controlling programs [93]. In a recent in vivo study in mice [94], AMPK regulation lipolytic activity in adipose tissue was confirmed, due to the direct phosphorylation of hormone sensitive lipase and adipose triglyceride lipase. AMPK as also been found to suppress lipogenesis by phosphorylating SREBP-1 c, inhibiting its transcriptional activity [95]. More recently, purebred IB foetuses have also been found associated with a downregulation of AMPK when compared to crossbred Iberian × Large White pigs in muscle [96]. Observed leptin overexpression would suggest an activated AMPK in AL, however an impaired leptin signalling as proposed by García-Contreras may also be occurring. IGF1 is the first cell response to GH stimulation. This anabolic factor contributes to cell differentiation and homeostasis though is not mandatory in adipose tissue development [97]. IGF1 is essential in the physical stress response that induce myocyte growth and hypertrophy [98], while regulating adipose tissue metabolism by inhibiting lipolysis similarly to insulin [99].

Another interesting canonical pathway found significantly affected by the DE genes ($p < 0.01$) include the complex TR/RXR of the nuclear retinoid X family receptors. This pathway is related to thyroid hormone activation, affecting key biological mechanisms such as differentiation, growth and development, lipid and carbohydrate metabolisms, thermogenesis and central nervous system functions [100]. In rats, thyroid hormone has been found to induce differentiation of preadipocytes in white adipose tissue, as well as to increase lipid content in cells, stimulating a variety of lipogenic enzymes including ACLY, ME1 and FASN, etc. [100]. However, in this canonical pathway, the software was not able to predict the direction of the activity pattern.

Other canonical pathways of interest found where no directional activity pattern was predicted included IL-6 and IL-9 signalling, the pentose phosphate pathway, Acetyl-CoA Biosynthesis III (from Citrate), Palmitate Biosynthesis I, Fatty Acid Biosynthesis Initiation II and Stearate Biosynthesis I. IL-6 is a pro-inflammatory cytokine, directly involved in increased fatty acid oxidation and decreased insulin resistance [101]. A group of interleukins, where IL-6 is included, can stimulate AMPK through the activation of signal transducer and activator of transcription 3 (STAT3) which was found upregulated in BI (log2 FC = −0.940, $q < 0.05$). On the other hand, IL-9 is a cytokine with an associated anti-inflammatory activity [102].

The main canonical pathway associated to the AL breed was Insulin receptor signalling (z-score = 1.633, $p < 0.05$). As mentioned before, insulin sensitivity plays an elemental role in modulating glucose and lipid deposition. Insulin accumulation induce triglyceride deposition while

inhibiting lipolysis culminating in more fat mass, where inflammatory cytokines tend to accumulate and promote more insulin resistance in the individuals.

Identification of potential upstream regulators and their effects at a transcriptional level to help explain the obtained DEG's was accomplished using IPA. A total of 643 regulator molecules were identified ($p < 0.05$, Supplementary Table S5) with 16 predicted to be active in AL (z-score ≥ 2) and 20 predicted to be active in BI (z-score ≤ -2).

Activated upstream regulators found in AL include mainly molecules associated with lipid homeostasis, adipogenesis and/or insulin sensitivity such as ADIPOQ, NR1D1, PPARG, SREBF2, SCAP and MED1. *ADIPOQ* is a gene exclusively expressed in the adipose tissue and its coding protein, adiponectin, is found circulating mostly within the plasma, where it participates in the regulation of several metabolic and hormonal mechanisms [103,104]. Adiponectin is known to trigger AMPK phosphorylation and increase the peroxisome proliferator-activated receptor alpha (PPARA) activity leading to β-oxidation, while being associated with reduced insulin resistance and anti-inflammatory activity through AdipoR1 and AdipoR2 signalling [104]. ADIPOQ circulating levels are inversely related with body fat mass and are higher when a low caloric intake is occurring [104]. In our trial, although animals from both breeds were slaughtered at ~150 kg of body weight, AL pigs have shown a higher ability to deposit fat mass, while BI developed more muscle mass [33], which suggest a higher expression of *ADIPOQ* in BI than in AL. On the other hand, our results indicate that ADIPOQ gene expression is not statistically different between breeds which would suggest that ADIPOQ activation as regulator could be a consequence of differential regulation at other levels, such as post-transcriptional regulation. NR1D1 has been previously described as a key factor in the coordination of the mammalian circadian rhythm with metabolic pathways in several tissues, including adipose tissue [105]. This transcription factor can stimulate adipogenesis and preadipocyte differentiation via a PPAR response element that induces increased expression of various adipogenic markers including *PPARG*, at a later adipogenesis stage [106,107].

The family of sterol regulatory element-binding proteins (SREBFs) is responsible for managing cellular lipid homeostasis in vertebrates by adjusting sterol-regulated lipogenic genes to the cell current needs [108]. SREBF2 is a transcriptional activator homolog to SREBF1 but, unlike the latter, is bounded to stimulate genes related to cholesterol synthesis such as *HMGCR*, *HMGCS*, *MVK* and *LDLR* and not fatty acid synthesis [108,109]. *SCAP* encodes for the protein responsible for the exportation of SREBF's to the Golgi complex for cholesterol synthesis by binding to the specific SREBF in the endoplasmic reticulum [110].

MED1 is a transcription factor of the Mediator complex and operates by mediating RNA polymerase II-dependent genes. Metabolically, MED1 is essential for adipocyte differentiation and adipogenesis by stimulating *PPARG* expression [111]. Adipogenesis comprehend two main steps. Firstly, early proadipogenic factors such as CEBPB, CEBPD and KLF's are stimulated and participate in the regulation of enhanceosomes formation which prompt the maturation of differentiated adipocytes by the activation of PPARG and CEBPA [112,113]. In this process, PPARG is recognized to have a master regulatory influence since the other factors cannot stimulate adipocyte differentiation without PPARG [112,114]. Upstream regulators associated to AL confirm the suggested hypothesis of an increased adipogenesis and cholesterol synthesis and uptake in this breed which agrees with the phenotypic data that characterises the fatter AL and the leaner BI genotypes [33,115].

Activated upstream regulators found in BI include molecules involved in the immunoregulatory and inflammatory processes such as MYD88, CCL2, IRF2, RELA and TNF, as well as regulators involved in lipid metabolism and insulin signalling such as INSIG1, INSIG2 and FOXO1. INSIG1 and INSIG2 are identical endoplasmic reticulum proteins and are responsible for mediating feedback control of sterol synthesis by binding to the SCAP protein transporter, preventing activated SREBPs transference to the Golgi apparatus to stimulate lipogenesis [116]. Furthermore, INSIG molecules can also bind to HMGCR, inhibiting its use of cytosolic acetyl-CoA for cholesterol biosynthesis [117]. FOXO1 is regularly expressed in insulin responsive cells such as the adipocytes, is involved in their differentiation

and can inhibit lipid synthesis by suppressing the transcriptional activity of PPARG [118]. This protein is also a suggested transcription factor with positive effect in myogenic growth, though studies are not consistent [119]. Other regulators also found to be associated with growth, myogenesis and muscle cell differentiation and proliferation were MEF2C and PDGFB.

Regulator effect analysis identified proliferation of fibroblast cell lines and activation of myeloid cells to be amongst the main functions associated with our determined upstream regulators. This is indicative of connective tissue development and an active state of the innate immune system, probably due to the obesity-associated inflammation, respectively. Additionally, regulator effect analysis together with some of the activated functions in BI may suggest a predominance of the stromal-vascular fraction in the cell population and not adipocytes. The complete list of master regulators and their effects on the regulators and genes in our dataset is shown in Table 2.

Table 2. Causal regulator effects predicted to be activated and inhibited in AL.

Master Regulator	Participating Regulators	Predicted Activation (AL)	Z-Score	*p*-Value	Target Molecules in Dataset
INSIG2	INSIG2	Inhibited	−2.236	3.74×10^{-6}	5
FLCN	Esrra, FLCN, MTORC1, PPARGC1A	Inhibited	−2.236	7.72×10^{-6}	20
PKIA	CREB1, Pka, PKIA	Inhibited	−2.887	1.26×10^{-5}	12
LRAT	Akt, INSR, JAK2, LRAT, RPE65, STAT5a/b	Inhibited	−3.300	3.03×10^{-5}	18
NRG4	NRG4	Inhibited	−2.000	3.76×10^{-5}	4
PDGF BB	PDGF BB	Inhibited	−2.333	1.54×10^{-4}	9
CREB1	CREB1	Inhibited	−2.828	1.69×10^{-4}	8
GNAS	GNAS	Inhibited	−2.236	1.97×10^{-4}	5
MFSD2A	MFSD2A	Inhibited	−2.000	4.85×10^{-4}	4
MEF2C	MEF2C	Inhibited	−2.236	1.47×10^{-3}	5
HSP27	Hsp27	Inhibited	−2.000	1.63×10^{-3}	4
NDFIP1	Akt, Jnk, Map3k7, NDFIP1, PTEN, SRC	Activated	2.449	2.31×10^{-6}	24
NR1D1	NR1D1	Activated	2.236	5.36×10^{-6}	5
NSUN3	NSUN3	Activated	2.000	5.61×10^{-6}	4
ALKBH1	ALKBH1	Activated	2.000	5.61×10^{-6}	4
UCHL3	Akt, AMPK, FOXO1, GSK3B, INSR, UCHL3	Activated	2.449	1.05×10^{-5}	24
MIR-29B-3P (AND OTHER MIRNAS W/SEED AGCACCA)	Akt, ERK, miR-29b-3p (and other miRNAs w/seed AGCACCA), PMP22	Activated	2.500	1.06×10^{-5}	16
ARNTL	Akt, ARNTL, CLOCK, NFE2L2	Activated	2.200	2.85×10^{-5}	25
IDH1	IDH1	Activated	2.236	2.97×10^{-5}	5
EPCAM	EPCAM	Activated	2.000	3.76×10^{-5}	4
ATP7B	Akt, ATP7B	Activated	2.887	4.58×10^{-5}	12
DAP3	DAP3	Activated	2.000	5.36×10^{-5}	4
BACE1	BACE1, CREB1, Pka	Activated	2.714	7.70×10^{-5}	11
ATP7B	ATP7B	Activated	2.236	3.98×10^{-4}	5
MLXIPL	MLXIPL	Activated	2.000	4.02×10^{-4}	4
SCAP	SCAP	Activated	2.449	4.21×10^{-4}	6
SREBF2	SREBF2	Activated	2.449	6.09×10^{-4}	6
GHRL	GHRL	Activated	2.000	8.06×10^{-4}	4
MED1	MED1	Activated	2.449	1.00×10^{-3}	6

Regulators and DE genes were used to conceive causal networks using IPA. One of the main networks established is related to lipid metabolism, carbohydrate metabolism and molecular transport (Figure 2). This network attained a total score of 31, summarizes the associated activity of 23 molecules and emphasizes the central role of *FASN* in the metabolic regulation in adipose tissue.

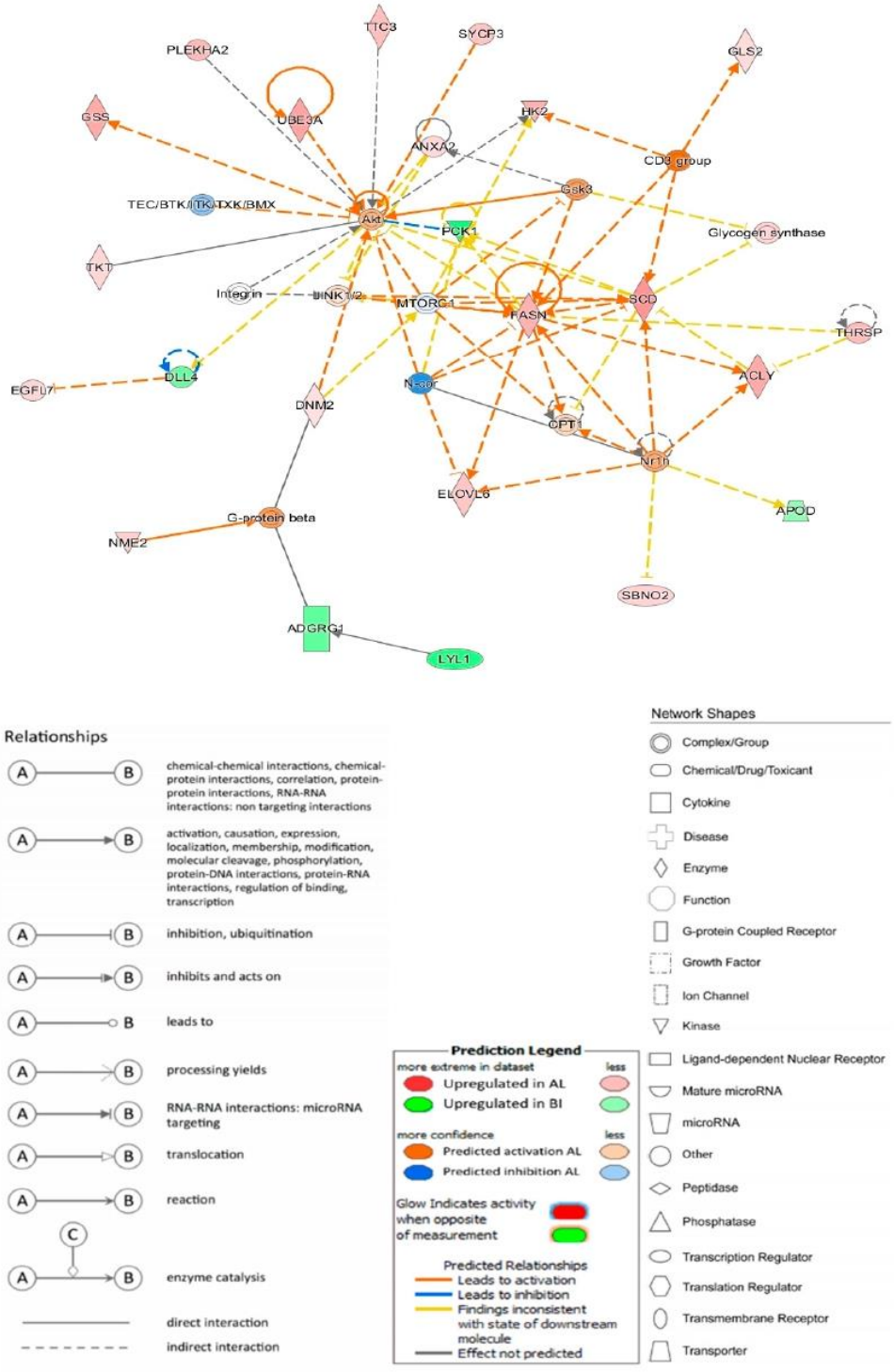

Figure 2. Carbohydrate metabolism, Lipid metabolism and Molecular transport Ingenuity Pathway Analysis (IPA) Network.

In normal cells, *de novo* lipid synthesis is only stimulated in case of lipid depletion, since these needs are usually met via absorption of dietary lipids. AL pigs are characterized by a genetic predisposition to be precociously adipogenic which, according to our data, may be associated to a deregulated *de novo* fatty acid synthesis leading to increased fatty acid accumulation, regardless of dietary lipid absorption.

In conclusion, the transcriptome analysis of the Alentejano and Bísaro Portuguese local pig breeds allowed the identification of a total of 458 differentially expressed genes and contributed with valuable insight to what is metabolically occurring in the adipose tissue of these breeds. Our data suggest that the determining factor that differentiate the higher fat deposition in AL is related to an increased *de novo* fatty acid synthesis, due to the upregulation of key genes such as *ACLY*, *FASN* and *ME1*. Cholesterol synthesis is also suggested to be higher in AL via SREBF2, SCAP and PPARG. Lipolytic activity may be more active in BI than AL through GH and AMPK signalling. Moreover, the predicted activation of INSIG1 and INSIG2 in BI suggests that this breed is more sensitive to insulin whereas the AL is less sensitive like the Iberian breed. Our data also suggest that AL pigs, such as previously described in Iberian pigs, have developed patterns of insulin and leptin resistance and obesity-induced chronic inflammation. Moreover, signalling of CD40 may play a dictating role in the development of insulin resistance in AL pigs.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/11/4/422/s1, Table S1: Primer design for qPCR, Table S2: List of differentially expressed genes, Table S3: Enriched biological functions in the set of differentially expressed genes, Table S4: Enriched canonical pathways in the set of differentially expressed genes, Table S5: Predicted upstream regulators for the set of differentially expressed genes.

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Chapter 5

Transcriptomic Profiling of Skeletal Muscle Reveals Candidate Genes Influencing Muscle Growth and Associated Lipid Composition in Portuguese Local Pig Breeds – article #4

Albuquerque, A.; Óvilo, C.; Núñez, Y.; Benítez, R.; López-Garcia, A.; García, F.; Félix, M.d.R.; Laranjo, M.; Charneca, R.; Martins, J.M. Transcriptomic Profiling of Skeletal Muscle Reveals Candidate Genes Influencing Muscle Growth and Associated Lipid Composition in Portuguese Local Pig Breeds. 2021. Animals, 11, 1423. https://doi.org/10.3390/ani11051423

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Note: The complete list of differentially expressed genes can be found in Appendix III.

animals

MDPI

Article

Transcriptomic Profiling of Skeletal Muscle Reveals Candidate Genes Influencing Muscle Growth and Associated Lipid Composition in Portuguese Local Pig Breeds

André Albuquerque [1,*], Cristina Óvilo [2], Yolanda Núñez [2], Rita Benítez [2], Adrián López-Garcia [2], Fabián García [2], Maria do Rosário Félix [3], Marta Laranjo [1], Rui Charneca [4] and José Manuel Martins [5,*]

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Simple Summary: Screening and interpretation of differentially expressed genes and associated biological pathways was conducted among experimental groups with divergent phenotypes providing valuable information about the metabolic events occurring and identification of candidate genes with major regulation roles. This comparative transcriptomic analysis includes the first RNA-seq analysis of the *Longissimus lumborum* muscle tissue from two Portuguese autochthonous pig breeds with different genetic backgrounds, Alentejano and Bísaro. Moreover, a complementary candidate gene approach was employed to analyse, by real time qPCR, the expression profile of relevant genes involved in lipid metabolism, and therefore with potential impacts on meat composition. This study contributes to explaining the biological basis of phenotypical differences occurring between breeds, particularly the ones related to meat quality traits that affect consumer interest.

Abstract: Gene expression is one of the main factors to influence meat quality by modulating fatty acid metabolism, composition, and deposition rates in muscle tissue. This study aimed to explore the transcriptomics of the *Longissimus lumborum* muscle in two local pig breeds with distinct genetic background using next-generation sequencing technology and Real-Time qPCR. RNA-seq yielded 49 differentially expressed genes between breeds, 34 overexpressed in the Alentejano (AL) and 15 in the Bísaro (BI) breed. Specific slow type myosin heavy chain components were associated with AL (MYH7) and BI (MYH3) pigs, while an overexpression of MAP3K14 in AL may be associated with their lower loin proportion, induced insulin resistance, and increased inflammatory response via NF*k*B activation. Overexpression of *RUFY1* in AL pigs may explain the higher intramuscular (IMF) content via higher GLUT4 recruitment and consequently higher glucose uptake that can be stored as fat. Several candidate genes for lipid metabolism, excluded in the RNA-seq analysis due to low counts, such as *ACLY*, *ADIPOQ*, *ELOVL6*, *LEP* and *ME1* were identified by qPCR as main gene factors defining the processes that influence meat composition and quality. These results agree with the fatter profile of the AL pig breed and adiponectin resistance can be postulated as responsible for the overexpression of MAP3K14's coding product NIK, failing to restore insulin sensitivity.

Keywords: Alentejano pig; Bísaro pig; transcriptome; skeletal muscle; meat quality; intramuscular fat; MYH3; MYH7; MAP3K14

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1. Introduction

Alentejano (AL) and Bísaro (BI) are the prevailing autochthonous pig breeds in Portugal. AL is reared in the southern region [1] and shares a genetic closeness with the Iberian pig [2], showing slow growth rates (excluding when under the late "montanheira" fattening regime) and an early and high adipogenic activity [3,4]. BI pigs, on the other hand, common to the northern region, belong to the Celtic line and share ancestors with leaner and highly productive breeds [5]. Furthermore, BI have a lower predisposition for (monounsaturated) fat accumulation when compared to AL pigs, but higher than most commercial lean breeds [6]. These two breeds are well-adapted to the environment, and their production chains provide high quality meat and fermented and dry-cured meat products [3,6].

Fatty acid content and composition are two of the most important factors that influence overall meat quality and consumer preference. A low saturated fatty acid (SFA) content is often desired because increases in this content have been found associated with raising blood cholesterol levels, particularly low-density lipoprotein cholesterol (LDL-c), increasing the risk of heart diseases [7]. On the other hand, increased monounsaturated fatty acid (MUFA) and essential polyunsaturated fatty acid (PUFA) contents are useful in decreasing LDL-c levels while increasing high density lipoprotein-cholesterol, and therefore reducing the risk of heart diseases [8]. Meanwhile, today's consumers are more aware of the specific nutritional value associated with meat, and that increased fat content contributes to better meat flavour while improving tenderness and juiciness, particularly when it occurs as intramuscular fat (IMF) at levels higher than 2.5% [9–11]. These fat stores can appear associated to intramuscular adipocytes or as droplets in the myofiber cytoplasm and can hold excess phospholipids, triacylglycerols, and cholesterol [12,13]. Although IMF content in AL pigs is generally higher than that in BI or other leaner highly productive breeds, their levels tend to fluctuate among several studies, ranging from 3 to 8%, mainly due to feeding and rearing conditions [3,14]. IMF content of the *Longissimus lumborum* muscle (LL) is determined and regulated by multiple metabolic pathways and is associated with the expression of genes involved in carbohydrate and lipid metabolism, cell communication, binding, response to stimulus, cell assembly, and organisation [15,16].

When compared to those of highly productive breeds, AL carcasses present a lower lean meat content (ranging from 37.5 to 51%) due to the above mentioned higher adipogenic activity and lipid deposition [3,14] while BI carcasses yield a moderate lean meat content (from 46 to 51%) [6]. In the *Longissimus lumborum* muscle, AL pigs present a high MUFA level (48–58%), particularly rich in oleic acid, and a low SFA content (35–44%) [3,17]. Studies regarding BI fat composition are scarce but show that MUFA levels (44–47%) [6,18] are lower and SFA levels (33–40%) similar or slightly lower than in AL pigs. Therefore, and when we consider the effects that unsaturated and saturated fatty acids have on consumers' health, BI pigs seem to display a slightly better balance of the unsaturated to saturated fatty acids ratio than AL pigs. On the other hand, higher PUFA levels are found in BI when compared to AL or improved genotypes, attaining 10% or higher [3,6,19].

RNA-seq experiments take advantage of next-generation sequencing developments to enable a quantitative screening for distinct gene expression patterns in individuals at the transcriptome level. Interpretation of differentially expressed genes (DEGs) and associated biological pathways provide valuable information about the metabolic events occurring and identification of candidate genes with major regulation roles. This comparative transcriptomic analysis includes the first RNA-seq analysis of the *Longissimus lumborum* muscle tissue from these two autochthonous pig breeds. Moreover, a complementary candidate gene approach was employed to analyse, by real time qPCR, the expression profile of relevant genes involved in lipid metabolism. This study can contribute to explain the biological basis of the phenotypical differences occurring between these breeds, particularly the ones related to meat quality traits that affect consumer interest.

2. Materials and Methods

The experiment was conducted in accordance with the regulations and ethical guidelines of the Portuguese Animal Nutrition and Welfare Commission (DGAV, Lisbon, Portugal) following the 2010/63/EU Directive. Staff members of the team involved in animal trials were certified for conducting live animal experiments by the Directorate of Animal Protection (DSPA, DGAV, Lisbon, Portugal).

2.1. Sampling and FA Profiling

Ten purebred male castrated AL and BI pigs (n = 5 for each breed) were reared in a traditional free ranged system and individually fed commercial diets at estimated ad libitum consumption from ~65 kg body weight until slaughter (~150 kg), as previously described [14]. *Longissimus lumborum* muscle samples were obtained at slaughter, snap frozen in liquid nitrogen and maintained at −80 °C until further use.

Total lipids were obtained using a Soxtherm automatic apparatus (S206 MK, Gerhardt). The respective FA profile was determined from the lipid extract, according to a previously described method [20]. After methylation [21], the FA samples were identified by GC-MS QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) and a 60 m × 0.25 mm × 0.2 µm fused silica capillary column (Supelco SP™ 2380, Baltimore, PA, USA). The chromatographic conditions were as follows: injector and detector temperatures were set at 250 and 280 °C, respectively; helium was used as the carrier gas at 1 mL/min constant flow; the initial oven temperature of 140 °C was held for 5 min, increased at 4 °C/min to 240 °C and held for 10 min. MS ion source temperature was set at 200 °C and interface temperature at 220 °C. Identification of FAMEs was based on the retention time of reference compounds.

2.2. RNA Extraction and Sequencing

Total RNA was isolated from 50–100 mg samples of LL following Ambion® RiboPure™ Kit (Thermo Fisher Scientific, Waltham, MA, USA) instructions. Total extracted quantity obtained was measured using NanoDropTM 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), while RNA quality was assessed with an Agilent 2100 BioanalyzerTM (Agilent Technologies, Santa Clara, CA, USA) following Agilent RNA 6000 Nano Kit instructions, along with NanoDropTM 1000 260/280 and 260/230 coefficients.

RIN values ranged from 7.8 to 9.0 with an average of 8.42. The total RNA was diluted into a concentration of 100 ng/µL and 3 µg were sent for stranded paired-end mRNA-seq sequencing in *Centro Nacional de Análisis Genómico* (CNAG-CRG, Barcelona, Spain) on a HiSeq2000 sequence analyser (Ilumina, Inc., San Diego, CA, USA). Currently, several RNA-seq experiments are performed at a low replication level and several publications suggest that a minimum of 2–3 replicates can be considered [22–24].

2.3. Quality Control, Mapping and Assembly

Generated Fastq files were analysed using FastQC (version 0.11.8) for quality confirmation [25] and Trim Galore (version 0.5.0) [26] was used to trim sequence reads for adapters, poli-A and poli-T tails. Low quality nucleotides (Phred Score, Q < 20) as well as short length reads (<40) were also removed, and the remaining reads were mapped to the reference pig genome version Sscrofa11.1 (Ensembl release 94) using HISAT2 version 2.1.0 [27]. Samtools−1.9 [28] was used to convert the obtained SAM files to BAM. Read counting and merging was performed with HTSeq-count version 0.11.1 [29].

2.4. Differential Expression Analysis

The obtained Gcount files were analysed using the R package DESeq2 [30], which estimates gene expression levels by counting total exon reads for the statistical analysis. Normalised counts were filtered for a minimum of 50 reads per group, and genes were considered as differently expressed when featuring an FDR lower than 0.05. The raw data shown and interpreted in this publication have been deposited in NCBI's Gene Expression Ombibus [31] and are available through the GEO Series accession number

GSE159817 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159817, accessed on 1 March 21).

2.5. Enrichment Analysis

An enrichment analysis based on the functional annotation of the differentially expressed genes was performed using the Ingenuity Pathways Analysis software (IPA; QIAGEN, Redwood City, CA, USA) to better understand their biological implications within the muscle tissue context. The list of DEGs ($q < 0.05$, $\log2FC \leq -0.7$ V $\log2FC \geq 0.7$) was uploaded into the software and converged with IPA's own library (Ingenuity Pathway Knowledge Base) to determine biologically pertinent information such as activated pathways, functions and regulators [32].

2.6. Real Time qPCR

Real Time quantitative PCR was performed to validate the data obtained by RNA sequencing (*MYH3*, *MYH7*, *TNNT1*, *MAP3K14*, *WDR91*, *FBXO32* and *FASN*) and explore other meat quality candidate genes outside the RNA-seq output (*ACACA*, *ACLY*, *ADIPOQ*, *ELOVL6*, *LEP*, *ME1* and *SCD*). Additional information on the selected primers can be found in the Supplementary Table S1. *MAP3K14*, *MYH3*, *TNNT1* and *WDR91* primers were designed using Primer3 version 0.4.0.

Previously extracted total RNA from the experimental animals was reverse transcribed in 20 µL reactions using Maxima® First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, MA, USA) following manufacturer's instructions.

The reaction mixes containing 12.5 µL of NZY qPCR Green Master Mix (2×) (NZYtech, Lisbon, Portugal), 0.3 µM of each sense primer and 12.5 ng of cDNA per sample were prepared in 96-well plates and run in a LineGene9600 Plus system (BIOER, Hangzhou, China). Standard PCRs were performed to confirm amplicon sizes, no-cDNA negative controls were performed within every plate and three replicates were performed per target sample. Cycling conditions included an initial 10 min holding denaturation stage at 95 °C, followed by 40 amplification cycles of 15 s denaturation at 95 °C and 50 s at 60 °C. To test PCR specificity a dissociation curve was added as a final step to the program comprising a single cycle at 95 °C (15 s) followed by 60 °C (60 s), and a ramp-up 0.2 °C/s to 95 °C for 15 s with acquired fluorescence. Moreover, PCR efficiency was predicted by standard curve calculation using five points of cDNA dilutions (1:4; 1:8; 1:16; 1:32; 1:64). *ACTB*, *HSPCB*, *RPL19* and *TOP2B* were the most stable genes tested using the geNorm software [33] and were, therefore, chosen as endogenous control genes for target normalisation ($m < 1.5$). Cycle Threshold values were regressed on the log of the previously constructed template cDNA curve.

2.7. Statistical Analysis

Results are presented as the mean ± SE. All data were tested for normality using the Shapiro–Wilk test. Individual data of growth, plasma, carcass and meat quality traits were analysed by one-way ANOVA with genotype as the main effect. For the carcass data, hot carcass weight was included as a covariate in the model. The SPSS Statistics software (IBM SPSS Statistics for Windows, v24.0; IBM Corp., Armonk, NY, USA) was used for data analysis. Mean differences were considered significant when $p < 0.05$, and values between 0.05 and 0.10 were considered trends.

To determine if the gene expression values were significantly different between the experimental groups, a student's t-test was executed using IBM SPSS Statistics software (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.) with an established significance level of $p < 0.05$. Equal variances of the samples were checked with Levene's Test for Equality of Variances with values lower 0.05 not considered as equal variances and another Independent Samples Test was performed assuming no equal variances. Equal variances were not assumed for *WDR91* ($F = 7.643$; $p = 0.024$) and *LEP* ($F = 8.929$; $p = 0.017$). Pearson correlation coefficients and associated *p*-values were also estimated.

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3. Results and Discussion

3.1. Productivity of Alentejano and Bísaro Breeds–Summary of Carcass Traits and FA Proportion

In previous studies, we assessed data of AL, BI and their reciprocal crosses, regarding their blood parameters, as well as their productive and meat quality traits, including the *Longissimus lumborum*. Briefly, BI showed significantly better carcass traits than AL and intermediate values were obtained in the crossed pigs (Table 1) [14].

Table 1. Zootechnical traits and physical–chemical parameters from muscle *Longissimus lumborum* of Alentejano (AL) and Bísaro (BI) pigs slaughtered at ~150 kg LW.

Trait	AL (n = 5)		BI (n = 5)		Significance
	Mean	SE	Mean	SE	
Days on trial	150.6	5.5	135.2	11.9	0.273
Average daily gain (g/day)	582.4	18.1	656.3	63.8	0.297
Feed conversion ratio (kg/kg)	5.45	0.21	4.3	0.47	0.039
Average backfat thickness (cm) *	7.9	0.4	4.3	0.3	<0.0001
Longissimus lumborum (%)	3.63	0.26	5.14	0.52	0.030
Moisture (g/100 g)	70.6	0.2	72.3	0.5	0.008
Total protein (g/100 g)	23.7	0.4	23.4	0.3	0.561
Total intramuscular fat (g/100 g)	7.3	0.2	5.7	0.2	0.001
Myoglobin content (mg/g)	0.83	0.12	0.43	0.04	0.014
Total collagen (mg/g DM)	13.7	0.6	16.3	0.7	0.025
pH (24 h post-mortem)	5.76	0.03	5.50	0.04	0.001
Drip loss (g/100 g)	0.55	0.07	2.25	0.21	<0.0001

Note: * Average of measurements taken between last cervical and first thoracic vertebrae (first rib), and last thoracic and first-lumbar vertebrae (last rib).

In this study, five randomly selected individuals from pure AL and BI breeds were chosen for transcriptome analysis through RNA-seq. AL pigs averaged a total of 150.6 days on trial with an average daily gain (ADG) of 582 g/d while BI averaged 135.2 days on trial ($p = 0.273$) and an ADG of 656 g/d ($p = 0.297$). At 150 kg and when compared to BI pigs, AL showed higher plasma total protein (69.8 vs. 64.4 g/L, $p < 0.05$), urea (6.9 vs. 5.6 mmol/L, $p < 0.05$) and total cholesterol (2.66 vs. 2.23 mmol/L, $p < 0.05$). Average backfat thickness was also significantly higher in AL (7.9 vs. 4.3 cm, $p < 0.001$) when compared to BI pigs. Regarding the LL muscle, AL pigs presented a lower loin proportion (3.63 vs. 5.14%, $p < 0.05$) but higher total IMF (7.3 vs. 5.7 g/100 g, $p < 0.01$) when compared to BI pigs. Several blood parameters from these pigs are listed in Tables S2 and S3.

The FA analysis identified oleic acid (C18:1 n-9) as the most represented fatty acid in the LL muscle of both breeds, with AL showing higher values when compared to BI (35.00 vs. 29.91%, respectively, $p < 0.05$). The total MUFA (C16:1 n-7, C18:1 n-7, C18:1 n-9, and C20:1 n-11) proportion was also higher in AL pigs (45.42 vs. 39.47%, $p < 0.05$). Regarding SFAs, palmitic acid (C16:0) was the most represented, with similar proportion in both breeds (22.28 vs. 21.22%, $p = 0.139$) while stearic acid (C18:0) was lower in AL pigs (10.77 vs. 12.38%, $p < 0.01$) when compared to BI. Total SFA (C10, C14, C16, C17, C18, and C20) proportion was also comparable between breeds (34.68 vs. 35.24%, $p = 0.587$). Finally, in respect to polyunsaturated fatty acids (PUFA), linoleic acid (C18:2 n-6) was the one most represented in both breeds, but a lower proportion was found in AL (12.06 vs. 15.17%, $p < 0.01$). Total PUFA (C18:2 n-6, C18:3 n-3, C18:3 n-6, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4 n-6, C22:5 n-3, and C22:5 n-6) proportion was also lower in AL when compared to BI pigs (19.90 vs. 25.29%, $p < 0.01$).

3.2. Mapping and Annotation

More than 750 million reads were initially obtained with an average number of sequenced reads over 39 million per sample. Read length was 76 base pairs. All samples shared an average associated quality score proximate to 40 and total GC content ranged from

53 to 56%. HISAT2 mapping of treated sequences was successfully achieved with an overall 97% of alignment rate, similar to a previous study conducted by our team with adipose tissue [34] but slightly higher than several previous pig transcriptome studies [35–40].

3.3. Differential Gene Expression with DESeq2 and qPCR Comparison

More than 25 k genes were firstly detected in the muscle tissue with around 3.6 k coding genes obtained after the initial filtering. The overall top five most expressed genes included rabaptin (RAB GTPase Binding Effector Protein 2, *RABEP2*), septin 1 (*SEPTIN1*), a non-identified coding gene (ENSSSCG00000036235), cathepsin F (*CTSF*) and SplA/ryanodine receptor domain and SOCS box containing 2 (*SPSB2*). These genes averaged a total read count per LL sample between 340 k (*RABEP2*) and 89 k (*SPSB2*).

A total of 49 genes were found to be differentially expressed (DE), with 34 being overexpressed in AL (log2 FC ≥ 0.7, $q < 0.05$) and 15 in BI (log2 FC ≤ -0.7, $q < 0.05$). In our LL samples, the most overexpressed gene in AL was the Cyclin and CBS domain divalent metal cation transport mediator 3 (*CNNM3*) (log2 FC = 4.487, $q < 0.01$), while in BI the most overexpressed gene was Stathmin 3 (*STMN3*) (log2 FC = −4.033, $q < 0.01$). The full detailed list of DEG's can be found in Table S4.

Myosin heavy chain 3 (*MYH3*) was found significantly overexpressed in BI in the RNAseq study (log2 FC = −1.191, $q < 0.05$) but only a numerical difference was observed when analysed through qPCR (log2 FC = −0.510, $p = 0.150$) (Figure 1). This gene is responsible for encoding part of a contractile protein, myosin, which is fundamental for the appropriate function of myofilaments, particularly the sarcomeres of striated/skeletal muscles. *MYH3* is also recognised as an embryonic myosin heavy chain, due to being mainly overexpressed during early mammalian development [41]. In mice, deletion of *MYH3* can induce postnatal changes in muscle fibre number, size, and type, while also deregulating other genes involved in muscle differentiation [41]. Within a species, muscle fibre composition is affected by genetics and several environmental factors while the different fibre types are associated with the four different myosin heavy chain isoforms, or their mixture [42,43]. In 2015, Hou [44] found significantly higher levels of MYH3 in leaner Landrace pigs when compared to fatter indigenous Chinese pig breeds. This author also suggested an association of the overexpression of *MYH3* with the larger diameter in muscle fibres of Landrace pigs. More recently, this gene was identified as a quantitative trait locus with considerable effect on IMF deposition, myofiber type differentiation and reddish meat colour (a^* from the CIELAB system) on skeletal muscle of Korean native pigs due to a variant in its promotor region [45]. The impact on a^* (red coloration) is mainly associated with the higher content of slow/type 1/oxidative myofibers which were, in turn, associated to an overexpression of the *MYH3* gene. BI's a^* values in LL samples are generally lower when compared to AL pigs (7.8 vs. 10.3, $p < 0.01$) [14] but slightly higher when compared to leaner breeds such as Landrace (5.63), Duroc (7.32) and Yorkshire (6.91) [46]. Similarly, and as mentioned before, BI pigs tend to accumulate less IMF than AL pigs (5.7 vs. 7.3 g/100 g, $p < 0.01$) but significantly more than extensively selected lean breeds. Therefore, our results indicate that in AL pigs other transcription regulatory mechanisms than MYH3 signalling play a bigger and more powerful role in influencing these complex traits since frequently meat quality traits depend on multiple mechanisms determined by numerous loci. Nevertheless, MYH3 signalling may explain the moderate to high levels of redness and IMF presented by BI pigs when compared to leaner breeds. These traits contribute to an improved meat quality and are due to possibly higher content of oxidative (red) muscle fibres, although muscle fibre types were not measured in this study.

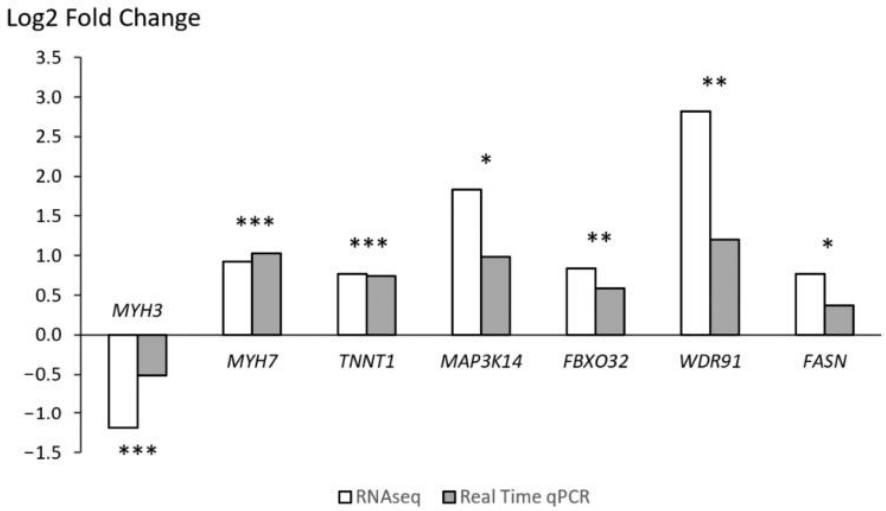

Figure 1. Gene expression comparison of *MYH3* (Myosin heavy chain 3), *MYH7* (Myosin heavy chain 7), *TNNT1* (Troponin T1), *MAP3K14* (Mitogen-activated protein kinase kinase kinase 14), *FBXO32* (F-Box Protein 32), *WDR91* (WD Repeat Domain 91) and *FASN* (fatty acid synthase) with RNA-seq and Real Time qPCR of the *Longissimus lumborum* of Alentejano (AL) and Bísaro (BI) pigs (n = 10). Positive values indicate overexpression in AL and negative values overexpression in BI. Pearson correlation values fluctuated between 0.93 (*MYH3*) and 0.58 (*FASN*). Significance of the correlation: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Surprisingly, a gene encoding for another myosin heavy chain component, *MYH7*, showed a trend towards AL (log2 FC = 0.921, q = 0.076), a difference boosted by qPCR (log2 FC = 1.025, $p < 0.01$). *MYH7* is also a molecular marker for slow/type 1/oxidative muscle fibres but is generally associated with the heavy chain subunit of cardiac myosin, although it can also be found expressed in skeletal muscle tissue [47].

Another important factor regulating muscle contraction is troponin T1 (*TNNT1*), a subunit of troponin which, as *MYH7*, tended to be overexpressed in AL either in RNA-seq (log2 FC = 0.764, q = 0.091) as in qPCR (log2 FC = 0.738, $p = 0.070$). *TNNT1* expression is also specific of slow skeletal muscle fibres in vertebrates and regulates muscle contraction through the troponin complex which is mediated by calcium concentration in the cells [48].

In our results, we associate both MYH7 as well as TNNT1 signalling in AL with an increase in the differentiation of slow muscle fibres, and to a stronger extent to what occurs in BI pigs through MYH3 signalling. No markers for fast skeletal muscle fibres were detected in the $p < 0.05$ or either $p < 0.1$ significance range suggesting identical presence of this fibre type in both breeds. On the other hand, red fibre type presence is suggested to be linked to MYH3 signalling in BI pigs, and MYH7 and TNNT1 signalling in AL pigs. Consequently, a higher presence of slow muscle fibres in AL pigs is associated with inducing reddish meat with increased IMF content and overall meat quality.

The SET and MYND Domain-Containing Protein 5 (SMYD5) is a member of the SMYD family of methyltransferases which play a crucial role in manipulating gene expression through post translational modifications on unique histone residues and other proteins [49]. SMYD proteins have been recognised as key regulators in skeletal and cardiac muscle development and function, though little is known about the specific roles linked to *SMYD5* [50]. Some studies also suggest a role of *SMYD5* in stimulating an anti-inflammatory response in *Drosophila* [51] and regulation of hematopoiesis in zebrafish [52]. In our data, *SMYD5* was found significantly overexpressed in BI pigs (log2 FC = −2.171, q < 0.01) which agree with the higher ability of this breed to increase muscle tissue when compared to AL pigs, as well as with our previously proposed obesity-induced chronic inflammation state, particularly in AL pigs [34].

Another determining factor of histone modification and transcriptional regulation found in our results was the gene encoding for lysine demethylase 2B (*KDM2B*), found overexpressed in AL pigs (log2 FC = 1.074, q < 0.01). KDM2B is generally associated with the demethylation of histones H3K4, H3K36, and H3K79, and repression of transcription. Recently, KDM2B was suggested to demethylate the non-histone target serum response factor (SRF), consequently inhibiting skeletal muscle cell differentiation and myogenesis [53]. On the other hand, KDM2B has also been heavily linked with an increased inflammatory response through epigenetic regulation of interleukin 6 [54]. Overexpression of *KDM2B* in AL pigs supports our previous hypotheses of lower muscle deposition and higher inflammatory state occurring in AL pigs when compared to BI pigs.

The lymphocyte specific protein 1 (LSP1) was found to be significantly overexpressed in BI pigs (log2 FC = −1.052, q < 0.01). *LSP1* encodes for an intracellular F-actin binding protein that participates in functions such as cell migration and signalling, particularly by regulating the recruitment of circulating leukocytes to inflamed sites [55]. In 2013, Ehrlich [56] first suggested the role of *LSP1* in influencing skeletal muscle development. Results from his work consistently associated an exceptional myogenic differential methylation in various subregions of the *LSP1* gene through binding to the myogenic transcription factor MYOD, particularly in the murine region of the gene. Consequently, DNA methylation status of LSP1 may prove key for alternative promoter usage and stimulating highly specific myogenic factors in BI pigs.

WD Repeat Domain 91 (*WDR91*) was found to be significantly overexpressed in AL pigs through RNAseq (log2 FC = 2.818, q < 0.01) as well as qPCR (log2 FC = 1.197, $p < 0.05$). This gene encodes for a protein that is known to negatively regulate a core subunit of the phosphoinositide 3-kinase (PI3K) complex. This complex is involved in the regulation of several functions including cell growth, proliferation, and differentiation. PI3K signals a network that ultimately leads to mTOR activation [57,58]. Continuous inhibition of this pathway in AL pigs suggests a decrease in overall protein synthesis which agrees with the lower muscle deposition when compared with BI pigs. Furthermore, PI3K activity can act as a molecular switch to regulate correct insulin signalling and activation [59]. Downregulation of this pathway through WDR91 agrees with the proposed lower insulin sensitivity in AL pigs via PI3K inhibition. *WDR91* has also been previously associated as a potential epigenetic regulator of skeletal muscle stem cells in adult mice, which are crucial for the maintenance and regeneration of adult skeletal muscles [60].

The role of epigenetics in the modulation of myogenesis is a current topic of increasing scientific interest, particularly due to the development of new methods that profile methylation. Mammalian DNA methylation is known to regulate the expression of specific target genes through silencing or upregulation, controlling the direction of major metabolic pathways [56]. Our results suggest a solid presence and influence of these mechanisms, particularly through activation of *SMYD5* and *LSP1* in BI and *KDM2B* and possibly *WDR91* in AL pigs, in stimulating BI muscle growth and inhibiting skeletal muscle differentiation in AL pigs, respectively.

The gene that encodes for the F-box protein 32 (*FBXO32*), also known as muscle atrophy F-box protein (*MAFbx*), tended to be overexpressed in AL pigs (log2 FC = 0.833, q = 0.094) which was confirmed by qPCR (log2 FC = 0.587, $p = 0.098$). Protein degradation in skeletal muscles is primarily mediated by the ubiquitin proteasome pathway, particularly muscle specific ubiquitin ligases, of which FBXO32 is proposed to have a central role in inducing proteolysis [61,62]. Increased skeletal muscle deposition demand higher protein levels which are dependent on the balance between protein synthesis and its degradation rates. Both, protein synthesis and proteolysis, are irreversible processes so that their resulting products, either proteins or aminoacids, do not influence the rates at which both processes take place [63,64]. Our results suggest a higher impact of proteolysis over protein synthesis in shifting this balance. Furthermore, overexpression of *FBXO32* is suggested to negatively modulate protein abundance on the skeletal muscle tissue in AL pigs and,

consequently, limit new muscle growth when compared to BI pigs, justifying the lower loin weight in AL pigs.

The mitogen-activated protein kinase kinase kinase 14 (*MAP3K14*) was to be found significantly overexpressed in AL with RNA-seq (log2 FC = 1.829, q < 0.01), a result also confirmed with qPCR (log2 FC = 0.983, $p < 0.05$). *MAP3K14* is a gene encoding for a serine/threonine protein-kinase (NF-κB-inducing kinase, NIK) that binds and transcriptionally regulates the expression of a number of molecules such as proinflammatory cytokines and chemokines [65]. High levels of MAP3K14 have previously been associated with skeletal muscle catabolism and atrophy [66], through increased expression of myostatin and decreased MyoD, which can be associated with the reduced loin proportion in the carcass of AL pigs when compared to that observed in BI (3.63 vs. 5.14%, $p < 0.05$). Several studies have also previously proposed the linkage of NIK overexpression with induced skeletal muscle insulin resistance and chronic inflammation development [67,68], in agreement with our previous suggestion of lower insulin sensitivity of the AL breed [34]. MAP3K14 has also been previously reported as a candidate gene to control feed efficiency in Duroc pigs [69] and its overexpression in AL agrees with the higher feed conversion ratios estimated in our AL and BI pigs (5.45 vs. 4.30 kg/kg, $p < 0.05$). Furthermore, in the hepatic tissue of obese mice, MAP3K14 has been also shown to reduce lipid oxidation via inhibition of peroxisome proliferator-activated receptor alpha (PPARA) [70]. While effects on the muscle tissue remain unclear in the current literature, a similar outcome occurring in the skeletal muscle of our pigs would agree with the fatter profile of AL's meat.

The ATPase H+ transporting V1 subunit C1 (*ATP6V1C1*) encodes for a component of vacuolar-type proton-translocating ATPase (V-ATPase) which is responsible for mediating the acidification of numerous intracellular components and was found to be overexpressed in AL pigs (log2 FC = 0.760, q = 0.046). This subunit C1 of V-ATPase is highly expressed in osteoclasts which participate in the breakdown of bone tissue [71] and may contribute to the higher bone mass found in BI when compared to AL pigs [72]. On the other hand, V-ATPase activity upregulated by ATP6V1C1 can activate the mTOR pathway which is involved in the regulation of multiple processes that lead to protein synthesis and muscle development [73].

Fibroblast growth factor (FGF) signalling can produce numerous beneficial effects on metabolic associated morbidities. FGFs are key for skeletal muscle regeneration and higher abundance is also associated with a higher presence of connective tissue [74,75]. FGFs signal via FGF receptors, requiring the binding of specific klotho proteins. Klotho Beta (*KLB*) is a cell-surface protein coding gene that was found overexpressed in AL pigs (log2 FC = 1.232, q < 0.01). The KLB product is suggested to be mandatory in the activation of several endocrine FGFs including FGF21, FGF19 and FGF15 [76]. FGF15 and 19 in particular are known to downregulate lipogenesis, bile acid metabolism and feeding response, while promoting cell proliferation [77]. On the other hand, FGF21 promotes insulin sensitivity, energy usage, and consequently weight loss [78]. In view of this, overexpression of KLB in AL pigs is, therefore, an intriguing result since AL pigs are proposed to have lower insulin sensitivity and higher lipid deposition than BI pigs. Nevertheless, other regulatory mechanisms might be influencing this pathway, particularly FGF receptor expression, which may play an important role in limiting FGF21, FGF19 and FGF15 signaling in the muscle tissue of AL pigs.

Surprisingly, the gene encoding for leiomodin 1 (*LMOD1*) which is associated with smooth muscle differentiation and contraction, has been found overexpressed in BI pigs (log2 FC = −0.714, q < 0.05) when compared to AL pigs. In vertebrates, LMOD2 and LMOD3 isoforms are preferably more expressed in skeletal muscle cells than LMOD1 [79]. Higher expression levels of the latter may be linked to hyperthyroidism while low levels have been linked to atherosclerosis in humans [80].

In our data, Casein kinase 1 delta (*CSNK1D*) was found overexpressed in BI pigs (log2 FC = −0.674, q < 0.05) when compared to AL pigs. *CSNK1D* participates in the regulation of several processes through phosphorylation of many distinct protein substrates involved

in cell proliferation and differentiation [81]. Additionally, it has also been demonstrated that CSNK1D is decisive in maintaining the accuracy of circadian rhythms in mammals [82]. The circadian clock is known to influence several canonical pathways including mTORC1 activity [83].

Regarding lipid metabolism related genes, prostaglandin E synthase 2 (*PTGES2*) was found to be significantly overexpressed in the fatter AL pigs (log2 FC = 1.551, q < 0.05). Prostaglandin E2 participates in several biological activities, including smooth muscle function, body temperature regulation, pain induction and stimulation of bone resorption [84]. PUFAs can affect prostaglandins by serving as substrates and competitive inhibitors for their synthesis [85]. PTGES2 mediates the synthesis of prostaglandin E2 from arachidonic acid (C20:4 n-6) which may explain the numerically higher proportion of this PUFA in BI pigs.

Prolyl 4-hydroxylase subunit beta (*P4HB*) was found overexpressed in AL pigs (log2 FC = 1.171, q < 0.05). *P4HB* encodes for a highly abundant and multifunctional protein involved in the catalysis of the formation, breakage, and rearrangement of disulphide bonds. Additionally, expression of P4HB has been linked with the biology of cytosolic lipid droplets in specific human enterocytes [86].

The gene encoding for 5′-aminolevulinate synthase 1 (*ALAS1*) was found significantly overexpressed in AL (log2 FC = 2.322, q < 0.05). The mitochondrial enzyme produced primarily catalyses the rate-limiting step in heme biosynthesis and its deficiency has previously been associated with acute hepatic porphyrias [87]. *ALAS1* is ubiquitously expressed, commonly regarded as a housekeeping gene, since every nucleated cell must synthesise a heme group for respiratory cytochromes [87]. More recently, ALAS1 has been associated with lipid metabolism regulation through peroxisome proliferator-activated receptor alpha (PPARA). A study by Rakhshandehroo [88] has demonstrated higher expression levels of *ALAS1* specifically induced by PPARA in human hepatocytes.

3.4. Functional Analysis

A total of 475 biological functions ($p < 0.05$) were retrieved by the IPA software for our dataset (Table 2). Four functions achieved a z-score enabling prediction of the activation direction, namely quantity of cells (z-score = 2.185) and quantity of leucocytes (z-score = 2.152), which were both predicted to be increased in AL, while neuronal cell death (z-score = −2.164) and apoptosis of tumour cell lines (z-score = −2.043) were predicted to be increased in BI.

As expected, *MAP3k14*′s coding product NIK is signalling the noncanonical NF-*k*B pathway, an alternative signalling cascade involved in the recruitment of leukocytes, macrophages, and lymphocytes. Though the respective retrieved z-score was below the threshold, lipid synthesis is suggested to be enhanced in AL which agrees with the previous phenotypical data.

A total of 64 upstream regulators were found related to the dataset ($p < 0.05$, Table S5) though none surpassed the required activation z-score threshold.

Ten networks associated to our gene dataset were identified with IPA. The top network found is represented in Figure 2, resuming 15 focus molecules and a total score of 3. This network illustrates the major involvement of the NF*κ*B complex (mediated by MAP3K14), Histone h3 complex (mediated by KDM2B), extracellular-signal-regulated protein kinase 1/2 (ERK 1/2, mediated by KLB) as main contributors for numerous gene interactions related to cell proliferation, differentiation and biochemistry.

Table 2. Top functions found with IPA associated with the target molecules in the Deseq2 dataset and their respective predicted activation state in Alentejano pigs (AL).

Functions	*p*-Value	Activation z-Score	Predicted Activation in AL	Target Molecules
Quantity of cells	3.64×10^{-2}	2.185	Increased	*IL12RB1, ITPR1, KDM2B, LSP1, MAP3K14, PSMB9, SPHK2, THRA, TRIB1*
Neuronal cell death	1.69×10^{-2}	−2.164	Decreased	*ITPR1, KDM2B, P4HB, SLC9A1, UCN*
Quantity of leukocytes	1.75×10^{-3}	2.152	Increased	*IL12RB1, ITPR1, LSP1, MAP3K14, PSMB9, SPHK2, THRA, TRIB1*
Apoptosis of tumour cell lines	1.32×10^{-2}	−2.043	Decreased	*ITPR1, KDM2B, LSP1, MAP3K14, P4HB, SLC9A1, SPHK2, THRA*
Binding of DNA	1.77×10^{-2}	1.993	-	*CBX1, MAP3K14, THRA, UCN*
Cell cycle progression	3.52×10^{-2}	1.964	-	*ANGEL2, CSNK1D, KDM2B, SLFN11, SPHK2, THRA*
Quantity of macrophages	1.08×10^{-3}	1.961	-	*IL12RB1, LSP1, THRA, TRIB1*
Quantity of B lymphocytes	9.65×10^{-3}	1.190	-	*ITPR1, MAP3K14, SPHK2, THRA*
Synthesis of lipid	3.57×10^{-2}	1.186	-	*MAP3K14, PTGES2, RUFY1, SPHK2, TRIB1*

3.5. Candidate Gene Expression Analysis with Real Time Quantitative PCR

A panel including the most relevant known genes involved in lipid metabolism was selected for quantification by RT-qPCR, as those were excluded in the RNAseq analysis due to their low reads counts. Results on these tested genes suggest a much more important role of lipid metabolism in defining the biochemical properties of each breed's muscle tissue (Figure 3) when compared to our RNA-seq results. Overall, lipogenic related genes were found significantly overexpressed in AL when compared to BI, such as in the cases of *ACLY* ($p < 0.01$), *ELOVL6* ($p < 0.01$), and *ME1* ($p = 0.01$), which agree with the previously mentioned higher IMF content of AL pigs' muscle tissue. A key gene in the de novo fatty acid synthesis, *FASN*, was only found with a numerical difference towards AL ($p = 0.115$), and the main fatty acid desaturation inducing gene, *SCD*, followed the same trend ($p = 0.131$). Similarly, expression levels of the complex multifunctional enzyme system coded by *ACACA* and responsible for catalysing the limiting step in fatty acid synthesis, was not statistically different between breeds ($p = 0.338$).

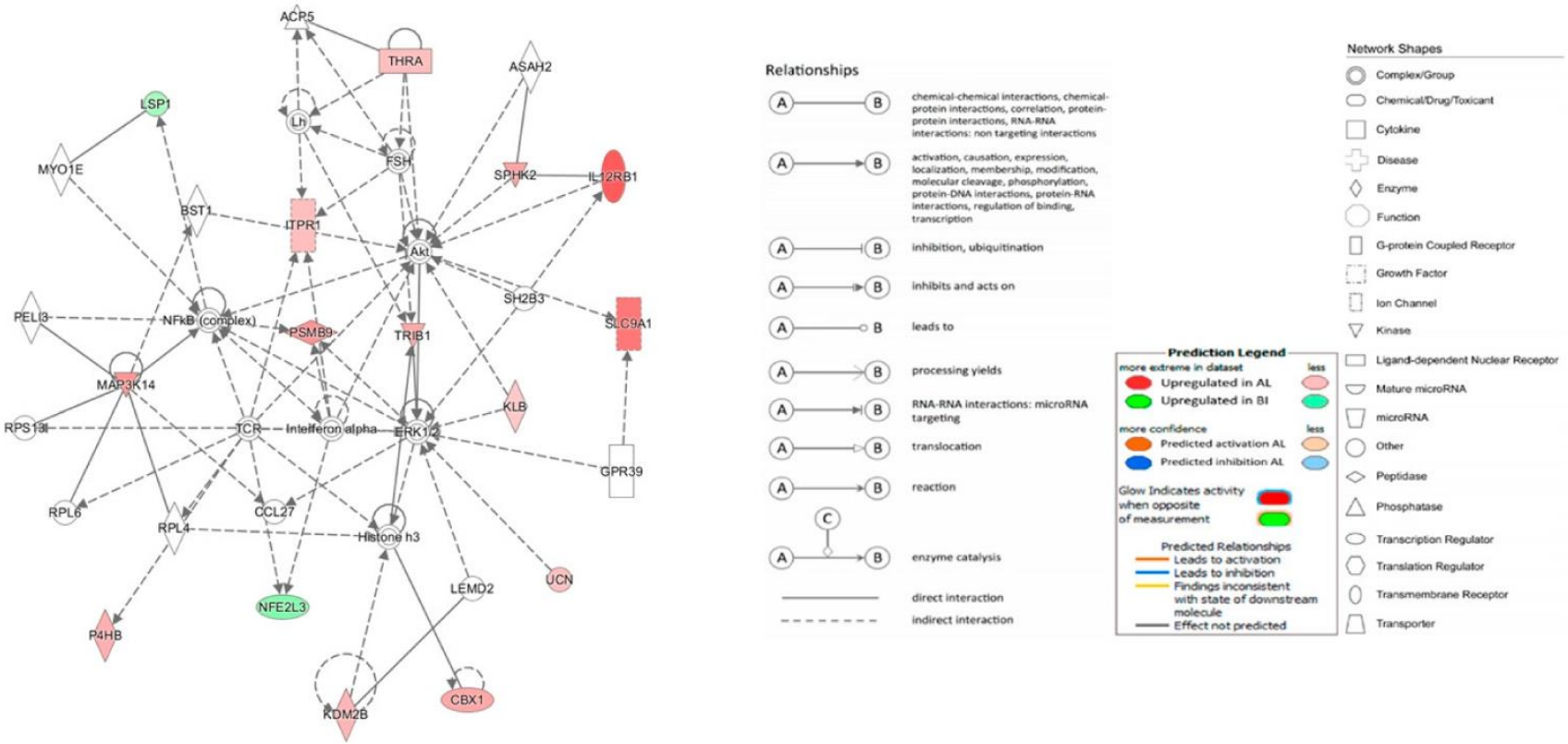

Figure 2. Cellular movement, lipid metabolism and small molecule biochemistry Ingenuity Pathway Analysis (IPA) Network.

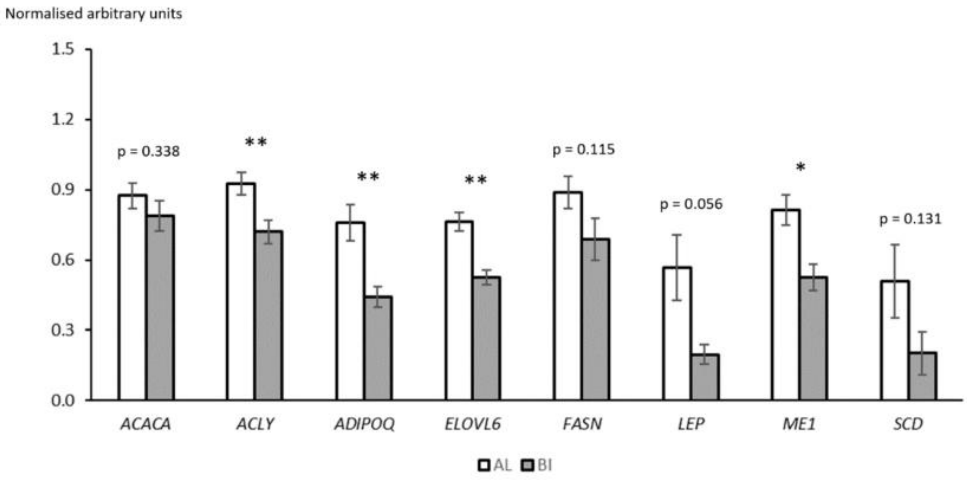

Figure 3. Estimated relative expression of candidate genes involved in lipid metabolism in the *Longissimus lumborum* of Alentejano (AL) and Bísaro (BI) pigs (n = 5 for each genotype) through Real Time qPCR. *ACACA* (Acetyl-CoA carboxylase alpha), *ACLY* (ATP citrate lyase), *ADIPOQ* (Adiponectin), *ELOVL6* (Elongation of long-chain fatty acids family member 6), *FASN* (Fatty acid synthase), *LEP* (Leptin), *ME1* (Malic enzyme 1), *SCD* (Stearoyl-CoA desaturase). Represented values are means of relative gene expression with their respective standard errors represented by vertical bars. Significance: ** $p < 0.01$, * $p < 0.05$.

Adiponectin, coded by *ADIPOQ*, is widely known to modulate the lipid and glucose metabolisms by activating fatty acid oxidation pathways and increasing blood glucose utilisation, which culminate in the activation of the AMPK pathway and an increase in energy supply [89]. A study by Choudhary [67] demonstrated that NIK overexpression can induce skeletal muscle insulin resistance, while ADIPOQ is responsible for suppressing NIK expression and restoring insulin sensitivity. In our dataset, the gene coding for NIK expression was found overexpressed in AL when compared to BI samples, suggesting an overcompensation in adiponectin levels. This was confirmed by the qPCR results (Log2 FC = 0.783, $p < 0.01$). These results suggest that adiponectin resistance is occurring, particularly in AL pigs, since higher ADIPOQ levels are associated with leaner individuals while lower levels of this cytokine are detected in obese individuals [90]. According to our RNAseq data, ADIPOQ receptors ADIPOR1 and ADIPOR2 were numerically higher in BI, without statistical significance. This may suggest that a potential lower signalling could be occurring in AL. Future investigation on the expression of these receptors, with a higher biological replication, as well as the circulating levels of adiponectin are needed to confirm this hypothesis.

Leptin, coded by *LEP*, is a cell-signalling hormone responsible for appetite regulation by informing the central nervous system when the total fat stored in the body rises. Individuals with high body fat composition exhibit higher levels of leptin, which signals the brain to decrease feeding and increase the use of stocked energy. Leptin is primarily secreted by the main energy store tissue of the body, white adipocytes, but it can also be found expressed in other tissues including skeletal muscle [91,92]. In our trial, LEP presented a tendency to be upregulated in AL pigs ($p = 0.056$) which agrees with the typically fatter composition of this breed when compared to BI. A suggested state of leptin resistance may be occurring in the AL, comparable to what happens with the genetically similar Iberian pig [93].

Our results from AL and BI breeds, particularly the differences in expression levels of lipogenic genes such as *ACLY* and *ME1* suggest that these may play an important role defining the synthesis of new fatty acids, overruling the importance of more central-rolled genes such as *ACACA* and *FASN*. ACLY and ME1 are responsible for catalysing reactions

that produce specific non-lipid precursors, including cytosolic acetyl-CoA and NADPH, that can later be used by ACACA and FASN to assemble palmitic acid (C16:0) [94,95]. This fatty acid is the main precursor for the synthesis of stearic acid (C18:0) through ELOVL6, and oleic acid (C18:1 n-9) through SCD. ELOVL6 is responsible for catalysing the reaction that introduces two carbon groups to several SFAs and MUFAs [96] while SCD is accountable for introducing a cis double bond at the delta-9 position in fatty acyl-CoA substrates, including stearic and palmitic acids [97]. Higher expression levels of ELOVL6 and SCD in the loin muscle agree with the previously mentioned higher oleic acid content of AL and may also justify the lower proportion of stearic acid (C18:0) when compared to BI. Nevertheless, we cannot exclude the possibility of more gene regulators being involved in influencing these traits, particularly ones related to fatty acid desaturation since SCD expression was only numerically higher in AL pigs. The generic overexpression of lipogenic related genes in the LL muscle of the AL breed agrees with the higher IMF content when compared to BI pigs. The contribution of lipolytic genes in the regulation of this metabolic balance remains unclear while the higher leptin and adiponectin signalling in the obese AL suggest that these hormones fail to stimulate lipolytic pathways, possibly through post-transcriptional regulation.

3.6. Linking Adipose and Skeletal Muscle Tissue Transcriptomes

In a previous study, we explored the genome function of these two local breeds at the adipose tissue level [34]. Several DE genes involved in lipid metabolism were previously detected in adipose tissue through RNAseq but were not detected in muscle tissue, including *ACLY*, *ELOVL6*, *FASN*, *LEP*, *ME1* and *SCD*. This agrees with the suggestion that lipid metabolism in muscle and adipose tissues are differently regulated [98,99].

Furthermore, the total DEG output ($p < 0.05$) was also much larger in the adipose tissue when compared to the muscle one (458 vs. 49, respectively), with a greater proportion found overexpressed in the AL breed (57 vs. 69%, respectively). This pattern agrees with the lower relevance of muscle tissue in influencing lipid composition through transcriptional and signalling regulation. Perception of adipose tissue as a mere energy storage element is outdated and its functions have been extended to a pivotal endocrine organ that secretes numerous substances that influence homeostasis and metabolism. On the other hand, the combining interactions between skeletal muscle cells and adipocytes have the most impact in defining fat and lean tissue depositions and their respective efficiency rates [100].

Both studies share a total of four DEGs in common, namely chromobox 1 (*CBX1*), integrator complex subunit 11 (*INTS11*), *STMN3* and RUN and FYVE domain containing 1 (*RUFY1*). *CBX1* encodes for a highly conserved protein that binds to methylated histone 3 tails at the lysine 9 residue, acting as an epigenetic agent that modulates chromatin structure and gene expression [101]. This gene was found to be consistently overexpressed in AL pigs in either adipose or LL (log2 FC = 0.954, $q < 0.05$ vs. log2 FC = 1.300, $q < 0.05$, respectively) which indicates the relevance of epigenetic mechanisms in regulating gene expression through histone modifications across different tissues, particularly in AL pigs. *INTS11* encodes for the integrator complex subunit 11, an element of the 12-subunit complex that participates in the transcription and processing of small nuclear RNAs [102] and was found consistently overexpressed in BI in both tissues (log2 FC = −1.297, $q < 0.01$ vs. log2 FC = −1.675, $q < 0.01$, respectively). On the other hand, *STMN3* encodes for a highly conserved phosphoprotein in vertebrates, generally associated with the deregulation of microtubule dynamics and tubulin sequestering [103]. *STMN3* is commonly associated with various functions in the nervous system and, in our results, presented similar overexpression patterns in BI pigs in both adipose tissue and LL (log2 FC = −2.058, $q < 0.01$ vs. log2 FC = −4.033, $q < 0.01$). *RUFY1* is a gene responsible for encoding a protein that binds to several signalling molecules and is suggested to participate in cell polarity and membrane trafficking mediated by small GTPases [104]. Curiously, *RUFY1* was found overexpressed in BI's adipose tissue and in AL's *longissimus lumborum* tissue (log2 FC = −1.809, $q < 0.01$ vs. log2 FC = 1.060, $q < 0.05$). This suggests that different

requirements are needed across tissues concerning *RUFY1*'s contribution towards cellular homeostasis. Additionally, *RUFY1*, also known as *RABIP4*, can modulate intracellular trafficking of the glucose transporter 4 (GLUT4) which plays a crucial role in increasing the transportation of circulating glucose to the cells [105]. Our results suggest that unexpectedly and despite similar circulating glucose levels in both breeds, either before or after slaughter (see Tables S2 and S3), on the adipose tissue higher circulating levels of glucose are taken to BI's adipocytes when compared to AL pigs, probably to be stored as fat after glucose homeostasis is achieved. This also suggests that the higher fat stores presented in AL when compared to BI pigs [14] are regulated through other mechanisms. On the other hand, in the LL the opposite is suggested to occur, where higher levels of glucose can be used as fuel to power the muscle cell or long-term stored as glycogen. Higher glucose uptake via *RUFY1* and *GLUT4*, in the AL's muscle tissue may explain the higher IMF content when compared to BI pigs.

Intramuscular fat participates in important regulatory roles in muscle, particularly through insulin-mediated glucose uptake and lipid peroxidation. Oversupply of fat stores is then strongly associated with decreased insulin sensitivity in skeletal muscle [106]. The proposed obesity-induced chronic inflammatory state in AL pigs, caused by exacerbated lipid accumulation, induces expression of pro-inflammatory cytokines and activation of numerous signalling pathways, including the nuclear factor-kappa B (NFκB) pathway, which ultimately are suggested to inhibit insulin signalling and action [107]. Interestingly, while we associated MAP3K14 with the activation of the NFκB pathway in the LL of AL pigs (log2 FC = 1.829, $q < 0.01$), in the adipose tissue this role seems to be played by MAP3K15 (log2 FC = 1.036, $q < 0.05$). However, development of insulin resistance involves several complex biological mechanisms that are not fully understood yet. BI's lower tendency to store fat when compared to AL pigs does not exclude the possibility of decreased insulin sensitivity to be occurring. BI pigs presented significantly higher total collagen content than AL pigs (13.7 vs. 16.3 mg/g, $p < 0.05$) which has previously been positively associated with insulin resistant skeletal muscle tissue in human patients [108].

Overall expression of the selected candidate genes involved in lipid metabolism was similar between adipose and muscle tissues. In short, lipogenic related genes were consistently more expressed in the AL breed across both studies which agrees with the fatter profile of this breed. Higher absolute fold change values were higher in adipose tissue though more significant differences between breeds were observed in the muscle tissue (Table 3).

Table 3. Candidate gene expression for lipid metabolism comparison between adipose tissue and *longissimus lumborum* in Alentejano (AL) and Bísaro (BI) pigs assessed with qPCR. Positive values indicate overexpression in AL and negative values overexpression in BI.

Genes	Adipose Tissue		*Longissimus lumborum*	
	log2 FC	*p*-Value	log2 FC	*p*-Value
ACACA	1.055	0.077	0.150	0.338
ACLY	1.601	0.068	0.362	0.017
ADIPOQ	−0.685	0.110	0.783	0.007
ELOVL6	0.671	0.136	0.540	0.001
FASN	1.359	0.100	0.367	0.115
LEP	0.929	0.046	1.524	0.056
ME1	1.008	0.106	0.627	0.010
SCD	1.351	0.087	1.325	0.131

Next-generation sequencing techniques such as RNA-seq provide a broad and insightful perspective over a tissue transcriptomics but still struggle with the analysis of low expression data, while differences within high read counts are more easily detected [109]. In our dataset, several key genes involved in lipid synthesis and regulation were found below the cut-off point regarding total read count in the filtering step, which determined

their premature withdrawal of the differential expression analysis. FASN, for example, in the adipose tissue scored a great correlation coefficient (0.92, $p < 0.01$) while in the muscle, where it was excluded in the initial filtering (<50 reads per group), presented a much lower, barely significant score (0.58, $p < 0.05$). This is why we suggest that genes with fewer reads detected through RNAseq should be accounted for with care since they arouse more associated noise and differential expression estimations may be biased against low read count values [110,111]. Besides the utility of RNA-seq at a standard read depth (30–60 million reads) for a general overview of the transcriptomic universe, Real Time qPCR provides a more reliable source of information to assess low expressed genes, as our results suggest.

In conclusion, this study intended to determine the nature of the biological events occurring in the muscle of the two main Portuguese autochthonous pig breeds and their interpretation regarding the phenotypical diversity shown by the two breeds. Our data allowed the identification of 49 differentially expressed genes through RNA-seq. Specific myosin heavy chain components have been associated with AL (MYH7) and BI (MYH3) pigs, while an overexpression of *MAP3K14* in AL may be associated with their lower loin proportion, induced insulin resistance, and increased inflammatory response. Our Real time qPCR data acknowledged the importance of several lipogenic genes and regulators, including *ACLY*, *ADIPOQ*, *ELOVL6*, *LEP* and *ME1*, disregarded in the RNA-seq by their lower total read count. These latter results agree with the fatter profile of the AL pig breed and adiponectin resistance may be responsible for the overexpression of MAP3K14's coding product NIK, failing to restore insulin sensitivity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11051423/s1, Table S1: Primer design for qPCR, Table S2: Plasma parameters from Alentejano (AL) and Bísaro (BI) pigs at ~150 kg LW, Table S3: Plasma parameters from Alentejano (AL) and Bísaro (BI) pigs slaughtered at 150 kg LW, Table S4: The full detailed list of DEGs, Table S5: Predicted upstream regulators for the set of differentially expressed genes.

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Data Availability Statement: The results from data analyses performed in this study are included in this article and its tables. Raw sequencing data are available through the GEO Series accession number GSE159817 or from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 6

General Discussion

6.1. Alentejano vs Bísaro: overall comparison of the genotype effect on the main growth, carcass, meat, and fat quality traits

There are scarce examples in the literature simultaneously comparing the productive performance of both breeds under the same conditions. To our knowledge, our works were the third ones to make such comparison. Focusing on our results of the Portuguese local pigs slaughtered at the end of the fattening period at approximately 150 kg BW, several data have to be highlighted. First, the AL genotype presented a slightly higher feed conversion ratio (5.2 vs 4.7 kg/kg; p=0.10) and lower ADG (602.8 vs 638.8 g/day; p=0.06) than the BI genotype. This confirms data from a previous work (Santos e Silva et al., 2000) where the authors state that AL pigs have a slow, less efficient growth when compared to BI. Furthermore, the hotter temperatures during summer in the Alentejo region, where the assay was conducted, may have limited the potential growth of BI pigs, which are used to colder summers in the northern regions of Portugal, and may have reduced the observed phenotypical differences between breeds due to heat stress. Both Portuguese local pig breeds presented lower growth rate estimations when comparing with modern lean breeds, which can attain more than 1000 g/day, when raised in intensive conditions (Čandek-Potokar et al., 2019).

Unsurprisingly, the levels of circulating total cholesterol (2.55 vs 2.06 mmol/L for AL and BI pigs respectively; p<0.01) and triacylglycerols (0.88 vs 0.71 mmol/L; p=0.08) were found higher in AL pigs when compared to BI. These results confirm the hyperlipidaemic profile of the AL genotype and, particularly, their higher genetic propensity to accumulate fat, since the provided diets and the average total feed consumption (data not shown) were identical for both genotypes. Total plasma protein levels were also significantly higher in AL pigs (78.0 vs 73.3 g/L; p<0.01) which suggests higher protein deposition in BI pigs. Finally, the lower observed plasma levels of cortisol in AL pigs (370.9 vs 492.6 nmol/L) suggests a higher stress response in BI pigs, which can also be associated with the aforementioned heat stress in BI pigs.

Carcass traits showed the distinct genetic potential of each pig breed. AL pigs presented shorter carcasses (88.7 vs 104.8 cm; p<0.01), lower head proportion (6.4 vs 7.4%; p<0.01) and lower bone cuts proportion (11.3 vs 13.7%, p<0.01) when compared to BI, supporting the claims of lighter bone structure of the former. Furthermore, when compared to BI pigs, carcass yield was slightly higher in AL (82.4 vs 81.1%) despite their significantly lower, but expected, commercial yield (46.5 vs 48.2%, p<0.05), which confirms this breed's great ability to accumulate fat, particularly at the subcutaneous and intermuscular levels, but limited muscle growth potential.

The chemical composition of both studied tissues, DSF and LL, demonstrated the influence of each genotype features. First, regarding DSF, the higher lipid fraction in AL when compared to BI (88.9 vs 83.7 g/100g; p<0.05) agrees with the high lipid content profile of this tissue (Sajic et al., 2011) and with the suggested higher fat accumulation profile in AL when

compared to BI pigs. On the other hand, a significantly lower total protein content was observed in AL when compared with BI pigs DSF (0.91 vs 1.29 g/100g; $p<0.01$). This can be due to differences in collagen content within the extracellular matrix of the adipose tissue (Pasarica et al., 2009) or associated to the number of adipocytes present per gram of adipose tissue. Proteins are the main backbone of many organelles (such as mitochondria), adipokines and membranes in cells. Although not done at this work, we can hypothesize that a histological study could reveal a higher number of adipocytes in BI and lower in AL, where hypertrophy could contribute to increase the size of the cells, allowing more fat accumulation within each adipocyte. Similarly, the fraction of each adipocyte type within our sampled tissue could provide useful information to better understand these results, since white adipose cells are significantly larger than brown or beige (Richard et al., 2000). In this way, AL pigs are suggested to present higher volume of white adipose tissue when compared to BI, which in turn might present higher proportions of brown and beige adipose tissue. Inconsistent claims of presence/absence of brown adipose tissue in pigs have risen a debate within the scientific community in the last decades (e.g. Attig et al., 2008; Hou et al., 2017; Meister et al., 1988). Piglets have previously been shown to possess a deletion between exons 3 and 5 in the *UCP1* gene that prevents its functional activity in operating the mechanism of non-shivering thermogenesis which naturally occurs in the brown adipose tissue of most mammals (Berg et al., 2006). However, other brown adipose tissue traits besides thermogenesis, including higher vascularization and increased numbers of both lipid droplets and mitochondrion, develop independently from UCP1 (Cannon and Nedergaard, 2010; Jastroch and Andersson, 2015; Ukropec et al., 2006). The lack of good thermoregulation in pigs have even been associated with the fact that wild pigs demonstrate greater care towards their offspring by huddling and nest building during cold times (Villanueva-García et al., 2021). Some authors also refer the importance of *UCP3* in the "browning" process of white adipose cells, since several breeds are known to better resist to cold temperatures and the better oxidative capacities of beige adipocytes may provide a reason to why this occurs (Lin et al., 2017). This also revokes the idea that UCP1 is the only thermogenic uncoupling protein.

Regarding DSF colour, significantly lower values for redness (2.25 vs 3.36, $p<0.05$) were obtained in AL comparing to BI pigs, which led to the lower chroma (5.0 vs 6.0, $p<0.05$) and saturation (0.06 vs 0.08, $p=0.05$) levels calculated in the former. Similarly, the significantly higher hue angle values (63.3 vs 56.4, $p<0.05$) in AL pigs can be explained by their lower redness values, particularly since the observed values for lightness (79.3 vs 79.1) and yellowness (4.47 vs 4.91) were not found to be significantly different between breeds. Furthermore, the higher lipid contents in the adipose tissue of AL pigs might be playing a role in diluting the blood vessels, lowering the redness and saturation readings.

Regarding the chemical properties of the LL muscle, it has to be highlighted that AL pigs showed significantly lower moisture content (71.0 vs 72.1 g/100g; $p<0.05$) but higher total IMF content (6.9 vs 6.0 g/100g; $p<0.05$) when compared to BI pigs. These results suggest higher juiciness and palatability of AL's meat and confirm the high adipogenic profile of this breed. On the other hand, total protein differences did not attain statistical significance (23.6 vs 23.0

g/100g; p=0.10). Myoglobin, the oxygen carrier which contributes for the red colour of meat due to the presence of haem pigment groups, was also found in significantly higher amounts in AL pigs (0.86 vs 0.45 mg/g; p<0.05), suggesting a more red, intense colour in this breed muscle when compared to BI. Total collagen content, however, was lower in AL pigs (13.1 vs 16.7 mg/g DM; p<0.01) suggesting the presence of less connective tissue in the muscle which can contribute to the higher overall tenderness of the meat. This higher tenderness was demonstrated to some extent by the WBSF test, which showed a tendency for lower values in the AL breed (41.9 vs 51.4 N, p=0.10) and can also be associated with the higher IMF content in these pigs, highly correlated with lower shear force values (Essén-Gustavsson et al., 1994). The measured ultimate pH (24h *post-mortem*) in both breeds was within the normal range in pork, which varies between 5.5 and 5.8 (Honikel, 1987). Nevertheless, AL pigs showed a significantly higher ultimate pH when compared with BI (5.72 vs 5.49, p<0.01). These results agree with the idea of a higher proportion of fast muscle fibres (type 2B) in the BI breed, which influences a lower pH due to the higher glycogen reserves in this fibre type (Bidner et al., 2004). In our study, AL pigs showed a significantly lower drip loss when compared to BI (0.59 vs 1.92 g/100g; p<0.01). The higher pH (Huff-Lonergan and Lonergan, 2005) and IMF content (Muriel et al., 2004) in AL pigs could positively influence water loss and explain the observed differences in drip loss between breeds. Finally, the measured colour of the LL muscle did not differ substantially, with the examples of redness (11.5 vs 10.9) or yellowness (3.18 vs 3.50) not attaining statistically significant differences, which led to similar values of chroma (11.9 vs 11.5) and hue angle (15.7 vs 18.1). The only exception was lightness, which was significantly lower in AL pigs (45.1 vs 50.0, p<0.01) and influenced their estimated higher saturation levels (0.27 vs 0.23, p<0.05) when compared with BI pigs.

6.2. Differential expression analysis in the adipose tissue

As suggested by several studies and our previous works (see chapters 2 and 3), increased carcass yield is mainly influenced by the fat deposition in adult pigs and, AL pigs in particular present a high and more precocious lipogenic profile than BI pigs. Consequently, changes in the expression of lipid metabolism related genes that would affect these processes should be expected. This is particularly true about AL pigs, where fattening is more prevailing than in BI. Therefore, genes directly related to lipogenesis and lipolysis that regulate the flow of nutrients and influence the rate of deposited fat were expected to display significantly different expression patterns between breeds. Finally, transcriptomic data associated with lipogenic/lipolytic genes in local pigs is limited in current literature (Poklukar et al., 2020) and it is essential and contemporary to characterize the genetic component of economically relevant production traits (Bendixen et al., 2010). This can be achieved by performing assays including genome-wide genotyping studies (Yang et al., 2013a), identification of new molecular markers using high density SNP chips for example (Muñoz et al., 2019) and through transcriptome profiling (Ayuso et al., 2015).

In this study comparing the transcriptome of the adipose tissue from the two most important Portuguese local pig breeds, we randomly selected a subset of 4x4 animals from the previous studies (see chapters 2 and 3) for RNA-sequencing. The average values obtained in this subset of animals followed the overall tendencies observed with the whole group, with the exception of the CIE colour values. In this case, and when comparing AL to BI, redness (2.49 vs 3.54), chroma (4.84 vs 6.02), hue angle (59.2 vs 55.3) and saturation (0.06 vs 0.08) changed their borderline significance (whole group) to non-significance (subset). The summary of the chemical composition and CIE colour parameters from DSF of the 4x4 subset can be found in Appendix IV. It must be highlighted the 6.9% higher total lipids in AL ($p=0.01$) and the 51.1% lower protein ($p<0.01$) when compared to BI pigs. Furthermore, the lipid profile of the subset can be found in Appendix V.

The FA analysis of the IMF fraction revealed oleic acid (C18:1 n-9) as the most represented FA in both breeds with AL presenting a significantly higher proportion when compared to BI (42.0 vs 37.7%, $p<0.01$). Consequently, total MUFA proportion was higher in AL pigs when compared to BI (49.4 vs 45.4%, $p<0.01$). Palmitic acid (C16:0) was the most represented SFA in both breeds although not attaining statistical differences between breeds (24.0 vs 25.4%, $p=0.27$). Stearic acid (C18:0) was the second most important SFA but also did not attain statistical differences between breeds (14.0 vs 14.0%, $p=0.95$). This led to a comparable total SFA proportion between breeds (40.2 vs 41.5%, $p=0.34$). Regarding PUFAs, linoleic acid (C18:2 n-6) was the most represented in both breeds, although with a significantly lower proportion in AL than in BI (8.6 vs 11.2%, $p<0.05$). Similarly, the total PUFAs proportion was significantly lower in AL when compared to BI pigs (10.5 vs 13.0%, $p<0.05$).

The transcriptomic analysis of the DSF of AL and BI yielded more than 660 million reads initially, averaging more than 41 million per sample per sense. After trimming, the number of reads used for mapping was over 650 million across all samples, 97.4% of the original size.

The obtained nucleotide sequences aligned between 96.52 and 97.19% with the pig reference genome used to map (Sscrofa11.1), a proportion higher than many transcriptomic studies previously completed (Ayuso et al., 2015; Benítez et al., 2019; Chen et al., 2011; Corominas et al., 2013; Puig-Oliveras et al., 2014; Ramayo-Caldas et al., 2012). This could be explained by an improved annotated genome version. The coding sequences matched over 10.7 K of genes, covering over 30% of the total number of genes in the pig genome (NCBI, 2022). Furthermore, we managed to identify a total of 458 DEGs in the two genotypes, with 263 genes overexpressed in AL pigs ($\log2\ FC \geq 0.6$, $q<0.05$) and 195 in BI ($\log2\ FC \leq -0.6$, $q<0.05$). Among them, 20% were novel genes (no associated description in most common databases).

Our results successfully identified several genes with known lipogenic action significantly more expressed in AL pigs, such as *ACLY* ($\log2\ FC = 1.853$, $q<0.01$), *FASN* ($\log2\ FC= 1.691$, $q<0.01$), *ELOVL6* ($\log2\ FC = 1.236$; $q<0.01$), *ME1* ($\log2\ FC = 2.090$, $q<0.01$) and *SCD* ($\log2\ FC = 2.311$, $q<0.026$). These genes directly participate in the cascade of events associated with the *de novo* lipid synthesis process, the elongation of very long FAs or their desaturation. *PCK1*, on the other hand, was found overexpressed in BI pigs ($\log2\ FC = -1.959$, $q<0.05$), suggesting that in these animals the gluconeogenesis/glyceroneogenesis pathway is favoured. This differs from what was observed in AL, where the lipid synthesis is preferred, leading to the accumulation of more

oxaloacetate. Higher amounts of oxaloacetate can boost malate concentrations and agree with the higher metabolization of pyruvate through *ME1* in AL, which provide more precursor NADPH molecules to meet their higher requirements for lipid synthesis in AL when compared to BI pigs. Other markers commonly associated with a more lipogenic profile that were found overexpressed in AL pigs include *LEP* (log2 FC = 1.376, q<0.05), *CEBPA* (log2 FC = 0.974, q<0.05) and *FABP4* (log2 FC = 1.061, q<0.01). Furthermore, our qPCR results generally confirmed the observations made through the RNA-seq experiment, with seven out of the nine selected target genes showing significative positive Pearson correlations (r≥0.71, p<0.05). The exceptions were *ELOVL6* and *SCD*, which presented only a statistical tendency (p=0.081 and p=0.086).

Cathepsin D, coded by the *CTSD* gene was found significantly overexpressed in the adipocytes of AL pigs (log2 FC = 0.91, q<0.01). *CTSD* activation has previously been studied in weight gaining mice and was found associated with hypertrophied adipocytes, inducing the activation of several proapoptotic proteins (Eguchi and Feldstein, 2013). Our results can support CTSD as a molecular marker for adipocyte hypertrophy and agrees with our previous suggestions of hypertrophied adipocytes in AL, opposing to a higher number of adipocytes in BI genotype.

A recent work by Benítez et al. (2019) studied the breed effect in the adipose tissue of IB (genetically similar to AL) and Duroc (leaner profile) pigs, and managed to identify several candidate genes that play relevant roles in cell growth and development which can also be found within our dataset. These genes include *IGF2* (log2 FC = -0.884, q<0.05), *FOS* (log2 FC = -1.087, q<0.05), and *FOSB* (log2 FC = -2.028, q<0.01) and were all consistently found overexpressed in the BI genotype, agreeing with the higher potential for growth of this breed. Other genes associated with cell proliferation, showed a similar expression pattern in our dataset suggesting that activation of growth and development factors can be achieved at a multiple tissue level, supporting the idea of the adipose tissue as an endocrine organ. The abovementioned genes include *APOD* (log2 FC = -1.087, q<0.05), *DUSP1* (log2 FC = -1.539, q<0.01), *EGR1* (log2 FC = -1.172, q<0.05), *ELN* (log2 FC = -1.743, q<0.01), *STAT3* (log2 FC = -0.940, q<0.05) and *KLF4* (log2 FC = -1.103, q<0.05). The last two mentioned, *STAT3* and *KLF4*, have also been studied for their specific effects in the adipose tissue. The members of the Signal Transducer and Activator of Transcription (STAT) family are able to regulate gene expression specifically to its target tissue. STAT3 for example, is suggested to play a regulatory role during preadipocyte differentiation. STAT factors are found in the cytosol until being recruited to a membrane receptor, where they will be tyrosine phosphorylated by a Janus kinase. Phosphorylated STAT factors then migrate to the nucleus where they can regulate the expression specific genes by binding to their DNA sequences (Mota de Sá et al., 2017; Richard et al., 2000). The Krüppel-like transcription factors (KLFs) include several members known to work together with other transcription factors to activate or inhibit transcription (Richard et al., 2000). Particularly, *KLF2* suppresses adipogenesis, while *KLF4* and *KLF6* promote adipogenesis (Mota de Sá et al., 2017). These genes were also upregulated in our dataset in BI pigs (*KLF2*, log2 FC = -1.236, q<0.01; *KLF4*, log2 FC = -1.103, q<0.05; *KLF6*, log2 FC = -1.439, q<0.05). Nevertheless, when comparing the base mean read count values for each of these three *KLF* (291.242 vs 147.955 vs 17.190, respectively), we understand that the adipogenic repressor KLF2 is much more expressed when compared to the promoters KLF4 and KLF6, which agrees with the lower adipogenic profile of the BI breed. Transcriptional repressors of adipogenesis including KLF2 and GATA2 display an ability to bind and inhibit PPARG

expression, the most influential transcriptional modulator of adipocyte development across all types of adipose tissue (Mota de Sá et al., 2017). In agreement with this, *GATA2* was also found overexpressed in BI (log2 FC = -1.120, q<0.05) when compared to AL pigs. Transcriptional factors of the GATA family bind to the consensus repeat DNA sequences 5'-AGATAG-3' to regulate the expression of numerous target genes, including *PPARG* and *CEBPs*. Additionally, several knockdown and overexpression studies suggest that GATA2 can suppress adipogenesis during preadipocyte differentiation in white adipose tissue (Mota de Sá et al., 2017; Tong et al., 2000).

Another growth factor found to be differentially expressed in our dataset, BMP6, is a member of the Bone Morphogenetic Proteins (BMPs). Although less studied than other members of this family, *BMP6* and its expression has been associated with increased brown adipose tissue, particularly during cold temperatures, and inducing brown adipocyte traits during preadipocyte differentiation (Mota de Sá et al., 2017; Richard et al., 2000). *BMP6* was significantly more expressed in AL pigs (log2 FC = 0.782, q<0.05) and curiously presented one of the highest base read means across all DEGs with 2 817.961, standing in the top 8% of the highest of all DEGs, and close to the top 1% of all detected genes. Comparing to other genes of interest to our study, only FASN showed a higher base mean value (3 861.632), standing in the top 6% of DEGs and 1% of all detected genes. Interestingly, the top DEG (148 289.264) encodes for glycogen synthase (*GYS1*), an enzyme responsible for catalysing the addition of glucose molecules to a glycogen backbone. *GYS1* also places third for highest average base of read counts when considering all detected genes in our study, with the first two being uncharacterized genes. Glycogen synthesis has recently been revealed as a key factor for the production of the multiple lipid droplets during brown adipose tissue differentiation at an early stage of life (Mayeuf-Louchart et al., 2019). AL pigs presented an overexpression of *GYS1* (log2 FC = 0.912, q<0.05) when compared to BI. Adipose tissue is known to contain low glycogen stores, and although the main roles of these in adipocyte metabolism remain uncertain, it is suggested that glycogen can function as a metabolic switch, regulating energy flow in coordination with lipogenesis and lipolysis (Markan et al., 2010).

Finally, it should be highlighted the overexpression of *MYH7* in BI (log2 FC = -2.397, q<0.05) when compared to AL pigs. A curious and often forgotten aspect about brown adipose tissue is that it can frequently exhibit muscle-like traits and previous studies have identified a functional link between brown adipose tissue and skeletal muscle (Farmer, 2008; Kong et al., 2018). One example of this is the expression of muscle-specific myosin heavy chains, such as the ones coded by *MYH7*, which appear to be exclusively expressed in brown adipocytes, with MYH7 knocked down expression resulting in reduced UCP1 expression during adipocyte differentiation (Tharp et al., 2018). However, conclusions of a higher proportion of this adipose tissue type in BI pigs cannot be taken lightly since the presence of these mRNA can also be a result of contamination of the adipose tissue by the stromal vascular fraction (Tharp et al., 2018). Additional studies regarding the cell type composition are required to confirm this hypothesis.

The functional analysis performed using the obtained DEGs allowed for the identification of several overrepresented biological functions, metabolic pathways, and regulator molecules.

A total of 380 genes were identified by the IPA database out of our original 458 DEGs. Exactly 500 different biological functions and diseases were found associated with our DEG dataset (p<0.05), although only 17 showed activation towards a breed (z-score ≤ -2 for BI or ≥ 2

for AL). Of these functions, 12 were activated in AL and these were mostly related with cell proliferation, cell infection and activation of granulocytes. On the other hand, 5 functions were activated in BI pigs, and these were mostly related to cell death and cell proliferation. Other functions identified that present a particular interest to our study include dyslipidaemia (z-score = -1.992, $p<0.01$, 11 genes), concentration of triacylglycerol (z-score = 1.415, $p<0.01$, 17 genes), synthesis of sterol (z-score = 1.387, $p<0.01$, 8 genes), quantity of white adipose tissue (z-score = 1.309, $p<0.01$, 7 genes) and concentration of long chain fatty acid (z-score = 1.223, $p<0.01$, 5 genes). These results establish the ability of AL pigs to synthesize and accumulate fat and agree with our previous results. Another function identified was inflammatory response (z-score = 1.861, $p<0.01$, 31 genes), which also agrees with the suggested chronic inflammatory state in the AL animals when compared to BI, due to their excessive fat accumulation.

A total of 392 canonical pathways were found associated to our gene dataset by the IPA software, although only 57 of these were found significantly enriched ($p<0.05$), and in only two the z-score values surpassed the required threshold to confirm a directional state (z-score ≤ -2 for BI or ≥ 2 for AL). The CD40 signalling pathway (z-score = -2.236, $p<0.01$, 5 genes) is one of these and previous studies in mice have related CD40 deficiency with obesity-induced insulin resistance and increased inflammatory responses (Guo et al., 2013; Yi and Bishop, 2014). These findings agree with the fatter profile of the AL pig, since excessive fat accumulation is highly associated with a higher release of pro-inflammatory molecules, which may have also developed a chronic low-grade inflammation state, and reduced insulin sensitivity (Poklukar et al., 2020). Other canonical pathways with interest include Growth Hormone Signalling (z-score = -0.378, $p<0.01$, 7 genes) which is known to activate lipolysis in adipose tissue when Growth Hormone receptors are stimulated (Kopchick et al., 2019). The Insulin Receptor Signalling pathway (z-score = 1.633, $p<0.05$, 6 genes) was one of the most relevant canonical pathways found in the direction of AL pigs and suggests that AL pigs may boost the production of insulin receptors in an attempt to compensate the reduced insulin sensitivity.

Regarding the activated upstream regulators found associated to our gene dataset, a total of 643 regulator molecules were identified ($p<0.05$), with 16 predicted to be active in AL (z-score ≥ 2) and 20 in BI (z-score ≤ 2). Upstream regulators activated in AL mostly consist of molecules related to lipid balance and insulin sensitivity such as PPARG (z-score = 2.742, $p<0.01$, 20 genes), ADIPOQ (z-score = 2.114, $p<0.01$, 9 genes), NR1D1 (z-score = 2.213, $p<0.01$, 5 genes), SREBF2 (z-score = 2.429, $p<0.01$, 6 genes), SCAP (z-score = 2.395, $p<0.01$, 8 genes) and MED1 (z-score = 2.415, $p<0.01$, 7 genes). PPARG in particular is widely known for its master regulatory influence in adipogenesis and adipocyte differentiation since other regulatory factors intervene in these metabolic processes through PPARG activation/inhibition (Rosen and MacDougald, 2006; Sarjeant and Stephens, 2012).

Many of the upstream regulators found activated in BI are associated with immunoregulatory and inflammatory processes. Moreover, other regulators detected are also involved with lipid metabolism and insulin signalling such as INSIG1 (z-score = -2.394, $p<0.01$, 6 genes), INSIG2 (z-score = -2.183, $p<0.01$, 5 genes) and FOXO1 (z-score = -2.202, $p<0.01$, 17 genes). INSIG1 and INSIG2 can negatively regulate cholesterol biosynthesis when induced by insulin (Dong et al., 2012), while FOXO1 is involved in adipocyte differentiation and can suppress the

transcriptional activity of PPARG, leading to lower lipogenesis (Junye et al., 2019). Growth hormone and IGF1 were very close to attain z-scores that indicate a directional activation, both predicted to be more expressed in BI pigs. Growth hormone (z-score = -1.766, $p<0.01$, 9 genes) is widely known for its anabolic and lipolytic properties and is directly mediated by the secretion of IGF1 (z-score = -1.869, $p<0.01$, 11 genes).

Our predicted regulator results agree with the suggested increased adipogenesis and cholesterol synthesis in AL pigs and also with the observed phenotypical results, namely the significantly higher total lipid content and fatter carcass profile of this breed when compared to BI.

However, the biological functions found by IPA associated to the dataset of regulators included cell death and proliferation (MFSD2A and SCAP as master regulators, 7 target DEGs and a consistency score of 5.292), activation of myeloid cells (ADIPOQ, ALKBH1, DAP3, NSUN3 and SIRT3 as master regulators, 10 target DEGs and a consistency score of 4.427) and proliferation of fibroblast cell lines (IDH1 as master regulator, 3 target DEGs and a consistency score of 0.577), among others. These results suggest a predominance of the stromal-vascular fraction within the adipose tissue. The stroma-vascular fraction includes all the other elements of adipose tissue excluding the adipocytes. This includes a collagen matrix, and nervous, blood and lymph cells, together with other elements such as preadipocytes, fibroblasts, monocytes, and macrophages (Martos-Moreno et al., 2013). Our results are also suggestive of connective tissue development and an active state of the innate immune system, possibly due to the obesity-induced chronic inflammatory state of the animals. An exacerbated and long-lasting inflammatory response could be triggering for example internal signals of adipocyte death, hypoxia, mechanical stress of the extracellular matrix due to the extensive tissue remodelling occurring (Poklukar et al., 2020). The higher recruitment of macrophages, granulocytes, and other immune cells, suggested by our results to be occurring in AL pigs, could be associated with a higher capacity of their adipocytes to store triacylglycerols. After the vital nutrient requirements are met, the excess energy is converted to triacylglycerols and stored within the common deposit locations such as the adipose depots under the skin. As these depots reach their storage limits, adipose tissue expands in size to try to accommodate more lipids and that is strongly associated with increased adipocyte death. When sensing these signals of cell death, pro-inflammatory macrophages are recruited to these locations to remove the remains in the damaged areas. These often lead to the secretion of more inflammatory adipokines, which usually fail to aid in obese subjects, gradually leading to a chronic inflammatory state and reduced insulin sensitivity (Richard et al., 2000).

A similar study to ours comparing two breeds with distinctive adiposity patterns (Basque vs Large White) (Vincent et al., 2012), suggested the occurrence of necrotic adipocyte death, mediated by macrophages, upon adipose tissue enlargement. Similar results were also obtained when comparing the adipose tissue of IB with that of Duroc pigs (lean phenotype). IB pigs, much like their genetically identical AL, presented an overexpression of genes associated with an increased inflammatory response, suggesting a chronic state of low grade inflammation (Benítez et al., 2019). The same IB pigs have also presented significantly higher levels of LEP, an hormone mostly expressed in adipocytes that control hunger, regulating feed intake and is known for stimulating lipolysis over lipogenesis (Stern et al., 2016). Our AL pigs, as much as these IB, have

presented significantly higher levels of LEP (log2 FC = 1.376, q<0.05) when compared to BI pigs. This suggests that, similarly to IB pigs, AL have naturally developed leptin resistance, enhancing the predisposition to overeat, become obese and develop metabolic syndrome.

6.3. Differential expression analysis in skeletal muscle

In this study comparing the transcriptome of the skeletal muscle LL between the two main Portuguese local pig breeds, we randomly selected a subset of 5x5 animals from the previous studies (see chapters 2 and 3) for RNA-sequencing. The average values obtained in the LL of this subset of animals reflected the overall tendencies observed with the whole group, with the exception of the hue angle values. In this case, and when comparing AL to BI, hue angle differences changed from NS (whole animals) to significantly lower in AL pigs (15.3 vs. 19.1, p=0.035 in the subset). The summary of the chemical composition, physical properties and CIE colour parameters from LL of the 5x5 subset can be found in Appendix VI.

It should be highlighted the 2.4% higher total moisture in BI pigs (70.6 vs 72.3 g/100 g, p<0.01) and, particularly, the IMF content which was found 1.6% higher in AL pigs (7.3 vs 5.7 %, p<0.01). The total protein fraction of the muscle was similar between breeds. However, BI pigs presented significantly higher LL muscle proportion in the carcass (3.63 vs 5.14 %, p<0.05).

The lipid profile of the subset can be found in Appendix VII. The FA analysis of the IMF fraction revealed oleic acid (C18:1 n-9) as the most represented FA in both breeds with AL presenting significantly higher proportion when compared to BI (35.0 vs 29.9%, p<0.05). Consequently, total MUFA proportion was higher in AL pigs when compared to BI (45.4 vs 39.5%, p<0.05). Palmitic acid (C16:0) was the most represented SFA in both breeds although not attaining statistical differences between breeds (22.3 vs 21.2%, p=0.139). Stearic acid (C18:0) was the second most important SFA and presented a significantly lower proportion in AL pigs (10.8 vs 12.4%, p<0.01). Meanwhile, total SFA proportion was comparable between breeds (34.7 vs 35.2%, p=0.587). Regarding PUFAs, linoleic acid (C18:2 n-6) was the most represented in both breeds, although with a significantly lower proportion in AL than in BI (12.1 vs 15.2%, p<0.01). Similarly, the total PUFAs proportion was significantly lower in AL pigs (19.9 vs 25.3%, p<0.01).

Approximately 781 million reads were initially obtained from the sequencing, averaging more than 39 million per sample per sense. After trimming, the number of reads used for mapping was slightly over 760 million across all samples, 97.3% of the original size.

The fragmented RNA sequences of our samples aligned between 96.46 and 97.19% with the pig reference genome used to map (Sscrofa11.1), a proportion very similar to what was obtained in our DSF study. The mapping results were better than many transcriptomic studies previously completed (Ayuso et al., 2015; Benítez et al., 2019; Chen et al., 2011; Corominas et al., 2013; Puig-Oliveras et al., 2014; Ramayo-Caldas et al., 2012), probably due to an improved annotated genome version. The coding sequences matched over 25.8 K genes, covering over 72% of the total number of genes in the pig genome (NCBI, 2022). Furthermore, we succeeded in identifying 49 DEGs, with 34 overexpressed in AL (log2 FC ≥ 0.7, q<0.05) and 15 in BI (log2 FC ≤ -

0.7, q<0.05). Among them, 10.2% are novel genes (no associated description in most common databases).

Our results allowed us to associate genes coding for specific slow type myosin heavy chain components, *MYH3* and *MYH7*, to each pig breed. *MYH3* encodes for an embryonic myosin heavy chain isoform and was found significantly overexpressed in BI pigs (log2 FC = -1.191, q<0.05), with the fourth highest read count average (720.183) among all DEGs, sitting in the top 2% across all detected genes. The muscles of adult pigs are known to express all four myosins heavy chain isoforms, and the proportion of these help determine the nature of muscle fibres 1, 2A, 2X and 2B (Lebedová et al., 2019; Lefaucheur et al., 2004). Furthermore, embryonic (MYH3 isoform) and early postnatal (MYH8 isoform) myosin heavy chain expression severely decreases after the first 2 to 4 weeks of life in rats, first in types 2B and 2X, followed by 2A muscle fibres (Schiaffino and Reggiani, 2011). However, expression of this myosin, coded by *MYH3*, is known to persist in adult life only in specific muscles in the eyes, jaw and in the intrafusal fibres of muscle spindles. Muscle spindles are common sensory structures in every muscle and are responsible for detecting sudden changes in fibre length (stretched and relaxed states, for example), reporting them to the central nervous system (Schiaffino et al., 2015). More recently, it has been suggested that developmental myosin heavy chains such as the embryonic and neonatal are re-expressed during muscle regeneration and can be used as molecular markers to determine regenerating fibres (Schiaffino, 2018; Schiaffino et al., 2015). Previous studies comparing leaner pig breeds to fat local genotypes, such as the one by Hou et al. (2016), have associated higher *MYH3* expression with the leaner breed and suggested a larger diameter in their muscle fibres. Other studies have uncovered a connection between *MYH3* expression and reddish meat (*a** from the CIELAB system), mainly due to a higher content of red slow/type 1/oxidative myofibers (Cho et al., 2019). The *MYH7* gene, encoding for another myosin heavy chain component (the cardiac isoform), showed an expressive trend towards overexpression in AL pigs (log2 FC = 0.921, q=0.076), which was enhanced during qPCR validation (log2 FC = 1.025, p<0.01). Similar to *MYH3*, *MYH7* stand in a top 2% position across all detected genes regarding the average read count, with 749.805 reads. Despite being generally associated with the heavy chain subunit of cardiac myosin, it can also be found expressed in skeletal muscle tissues where it's a marker for slow type 1 muscle fibres (Schiaffino et al., 2015). Another molecular marker found associated with AL LL muscle was *TNNT1* (log2 FC = 0.764, q = 0.091). This gene encodes for a subunit of troponin, troponin T1, which regulates muscle contraction mediated by calcium concentrations in the muscle cell. The troponin complex is formed of three distinct subunits: C, which binds calcium; T, which binds tropomyosin; and I, which is an inhibitory subunit (Wei and Jin, 2016). Higher expression values of TNNT1 may suggest that the *rigor mortis* process in the LL of AL, started with the binding of the actin-myosin complex, occurs in a less expressive way. This could induce less shortening of the myofibers, reducing the hardness of the meat associated with it. This agrees with the results of the WBSF test obtained in the LL samples of both genotypes, which presented almost 30% lower values for the AL breed when compared with BI (p = 0.072).

Our results allow us to associate both MYH7 as well as TNNT1 signalling in AL with an increase in the differentiation of slow muscle fibres, while in BI pigs that seems to be achieved through MYH3 signalling. Furthermore, the redness (*a**) measured in LL of both breeds indicated over 17.5% higher values in AL pigs, although not attaining statistical significance (11.4 vs 9.7,

p=0.147 in the subset) which may suggest a slightly higher content of type 1 fibres in this breed. This fact also suggests that both MYH7 and TNNT1 (or their combination) might be more important molecular markers for red myofibers than MYH3. However, both breeds still present an average of redness values much higher than the ones observed in leaner breeds such as Landrace (5.63), Duroc (7.32) and Yorkshire (6.91) (Choi et al., 2016). The suggested higher presence of slow muscle fibres in AL pigs also agrees with the increased IMF content and overall meat quality in this breed. Nevertheless, histological studies should be carried out to confirm such hypothesis. Additionally, no markers for fast skeletal muscle fibres were detected in the $p < 0.1$ significance range, suggesting identical signalling and presence of this fibre types in both breeds.

The role of epigenetics in the modulation of myogenesis is a current topic of scientific interest, particularly due to the development of new methods that profile methylation. Mammalian DNA methylation, for example, is known to regulate the expression of target genes through silencing or upregulation, controlling the direction of major metabolic pathways (Ehrlich and Lacey, 2013). Our results suggest a solid presence and influence of these mechanisms, particularly through activation of KDM2B (log2 FC = 1.074, q<0.01) and WDR91 (log2 FC = 2.818, q<0.01) in AL pigs and SMYD5 (log2 FC = -2.171, q<0.01) and LSP1 (log2 FC = -1.052, q<0.01) in BI, in suppressing skeletal muscle differentiation in AL pigs and stimulating BI muscle growth. However, transcriptional regulation is usually the result of complex interactions of the various regulatory factors with the genome, thus it is essential to perform additional studies, for example of histone modification and DNA methylation, besides analysing gene transcription (Frese et al., 2013).

Identification of genes directly influencing protein metabolism, particularly proteolysis, is of the utmost importance since these affect the process converting muscle to meat and its associated quality features. The gene that encodes for the F-box protein 32 (*FBXO32*), also known as muscle atrophy F-box protein (*MAFbx*), tended to be overexpressed in AL pigs (log2 FC = 0.833, q=0.094), which was later confirmed by qPCR (log2 FC = 0.587, p=0.098). Protein degradation in skeletal muscles is primarily mediated by the ubiquitin proteasome pathway, particularly muscle specific ubiquitin ligases, of which FBXO32 is proposed to have a central role in inducing proteolysis (Li et al., 2016b; Mascher et al., 2008). Increased skeletal muscle deposition demand higher protein levels which are dependent on the balance between protein synthesis and its degradation rates. Both, protein synthesis and proteolysis, are irreversible processes in a way that their resulting products, either proteins or amino acids, do not influence the rates at which both processes take place (Duan et al., 2016; Rothman, 2010). Our results in AL pigs suggest a higher impact of proteolysis over protein synthesis in shifting this balance. Furthermore, overexpression of *FBXO32* is suggested to negatively modulate protein abundance on the skeletal muscle tissue of this genotype, although the total protein content between breeds did not differ. Consequently, this will limit new muscle growth when compared to BI pigs, agreeing with the lower trimmed ham, loin and commercial yield proportions in AL pigs (12.1 vs 16.0%, p=0.014; 3.63 vs 5.14%, p=0.03; and 45.4 vs 48.2%, p=0.095).

Thyroid hormones play an important role in cell growth, differentiation, and metabolism in skeletal muscles, with the expression of its receptors, either *THRA* (thyroid hormone receptor

alpha) or *THRB* (thyroid hormone receptor beta) mediating their action (Bloise et al., 2018). *THRA* was found significantly more expressed in AL pigs when compared to BI (log2 FC = 0.954, q<0.05) and presented the highest average read count among DEGs with 3 214.382, sitting in the top 0.4% among all detected genes. Despite crucial for myogenesis and proper cell function, excess of thyroid hormones can increase protein degradation and even lead to muscle atrophy (Carter et al., 1980; De Stefano et al., 2021), which would agree with the lower muscle development observed in AL pigs, when compared with BI.

The mitogen-activated protein kinase kinase kinase 14 (*MAP3K14*) was also found significantly overexpressed in AL in our RNA-seq results (log2 FC = 1.829, q<0.01). *MAP3K14* encodes for a serine/threonine protein-kinase (NF-κB-inducing kinase, NIK) that transcriptionally regulates the expression of several proinflammatory molecules such as cytokines and chemokines, increasing their local recruitment (Liu et al., 2012). Higher levels of MAP3K14 have previously been associated with skeletal muscle degradation and atrophy (Fry et al., 2016), through increased expression of myostatin and decreased MyoD, which agree with the previously mentioned reduced loin proportion of AL pigs when compared to BI. Furthermore, several studies have also proposed the linkage between NIK overexpression and induced skeletal muscle insulin resistance and chronic inflammation (e.g. Barma et al., 2009; Choudhary et al., 2011), in agreement with our suggestion of lower insulin sensitivity in AL pigs. *MAP3K14* has also been previously reported as a candidate gene for feed conversion ratio in Duroc pigs due to two significant SNPs found (Ding et al., 2017). Finally, in a study in the hepatic tissue of obese mice, MAP3K14 has also been shown to reduce lipid oxidation via inhibition of peroxisome proliferator-activated receptor alpha (PPARA) (Li et al., 2020). While the effects on the muscle tissue remain unclear in the current literature, a similar outcome occurring in the skeletal muscle of our pigs would agree with the fatter profile of AL's meat.

The ATPase H+ transporting V1 subunit C1 (*ATP6V1C1*) encodes for a component of the vacuolar-type proton-translocating ATPase (V-ATPase) responsible for mediating the acidification of various intracellular components. This gene was found overexpressed in AL pigs (log2 FC = 0.760, q<0.05) and this C1 subunit of V-ATPase is known for being highly expressed in osteoclasts which participate in the breakdown of bone tissue (Feng et al., 2009). This agrees with the lower bone mass found in AL pigs when compared to BI (Martins et al., 2020). On the other hand, V-ATPase activity upregulated by ATP6V1C1 can activate the mTOR pathway which is involved in the regulation of multiple processes associated to protein synthesis, muscle development and skeletal muscle fibre type composition (McConnell et al., 2017).

One of the few DEGs of our dataset directly associated with the lipid and glucose metabolisms was the RUN and FYVE domain containing 1 (*RUFY1*). RUFY1 is a GTPase-activating protein that binds to several signalling molecules and can also modulate the intracellular passage of glucose transporter 4 (GLUT4), which plays a crucial role in the transportation of circulating glucose to the cells through facilitated diffusion (Kitagishi and Matsuda, 2013; Mari et al., 2006). *RUFY1* was found overexpressed in AL pigs (log2 FC = 1.060, q<0.05), suggesting higher glucose uptake in AL pigs from the blood into the muscle cells, where it can be used to obtain energy, can be stored as glycogen, or be converted into FAs to increase IMF stores. The latter may contribute

to explain the observed higher IMF content in AL's skeletal muscle tissue when compared to BI pigs.

The functional analysis performed using the IPA software and the subset of obtained DEGs allowed for the identification of some overrepresented biological functions, metabolic pathways, and regulatory molecules. Out of our original 49 DEGs, a total of 37 genes were identified by the IPA database associated with 475 different biological functions and diseases associated ($p<0.05$). However, only four of these presented enough z-score to allow a directional activation, namely quantity of cells (z-score = 2.185, $p<0.05$, 9 genes) and quantity of leukocytes (z-score = 2.152, $p<0.01$, 8 genes) for the AL genotype, and neuronal cell death (z-score = -2.164, $p<0.05$, 5 genes) and apoptosis of tumour cell lines (z-score = -2.043, $p<0.05$, 9 genes) for the BI genotype. Other biological functions of interest included quantity of macrophages (z-score = 1.961, $p<0.01$, 4 genes), quantity of B lymphocytes (z-score = 1.190, $p<0.05$, 4 genes) and synthesis of lipid (z-score = 1.186, $p<0.05$, 5 genes). These results agree with our hypothesis, *MAP3k14*'s coding product NIK is signalling the noncanonical NF-kB pathway, an alternative signalling cascade involved in the recruitment of leukocytes, macrophages, and lymphocytes. They also agree with the suggested chronic inflammatory state in AL pigs. Furthermore, myoglobin is also a known muscle damage marker (Brancaccio et al., 2010) and the significantly higher levels of myoglobin found in the LL muscle of AL pigs, when compared to BI, can also be associated with a higher inflammatory response.

A total of 137 canonical pathways (132 enriched, $p<0.05$) and 64 upstream regulators ($p<0.01$) were associated with our subset, although none surpassed the minimum activation z-score threshold, probably due to the low number of DEGs.

Our qPCR results generally confirmed the observations made through the RNA-seq experiment, with all 7 target genes selected for validation showing significative positive Pearson correlations ($r\geq0.58$, $p<0.05$).

Regarding the lipid metabolism events occurring within the LL muscle, and since our RNA-seq results were limited regarding these pathways, a panel including the most relevant genes involved in lipid processes was added to the set of genes selected for validation through qPCR. These genes included *ACACA*, *ACLY*, *ADIPOQ*, *ELOVL6*, *FASN*, *LEP*, *ME1* and *SCD*. Most of these have been excluded by the RNA-seq analysis when using the R package DESeq2 and applying traditional filtering options (50 reads per group). However, it is known that genes with fewer reads detected through RNA-seq should be considered with care since they arouse more associated noise, and differential expression estimations may be biased against low read count values (Esteve-Codina, 2018; Raithel et al., 2016). Furthermore, RNA-seq experiments at a standard read depth (30-60 million per sample) such as ours, focus on providing a general overview of the present transcriptomic universe at that given timepoint, with RT-qPCR providing a more reliable tool to assess low expressed genes. Overall, the results of the qPCR recognized the role of lipogenic genes in AL, which are suggested to portray the higher IMF content observed in the LL muscle of this breed when compared to the leaner BI. This is particularly true regarding the overexpression of genes encoding for the main non-lipid precursors of the *de novo* lipogenesis (cytosolic acetyl-CoA and NADPH, respectively) such as *ACLY* (log2 FC = 0.362, $p<0.05$) and *ME1* (log2 FC = 0.627, $p=0.01$). Furthermore, *ELOVL6*, responsible for catalysing the

elongation of several lipid precursors, was also found significantly more expressed in AL pigs (log2 FC = 0.540, p<0.01) when compared to BI. Surprisingly, both *FASN* and *SCD*, only showed numerical differences (p=0.115 and p=0.31, respectively) while *LEP* presented a strong tendency for AL pigs (log2 FC = 1.524, p=0.056). Higher levels of ELOVL6 and SCD in the LL muscle agree with the observed higher oleic acid content of AL and may also explain the lower proportion of stearic acid (C18:0) when compared to BI. However, we cannot exclude the possibility of other gene regulators influencing these traits, particularly the ones related to FA desaturation since *SCD* expression was only numerically higher in AL pigs. The general overexpression of lipogenic genes in the LL muscle of the AL breed agrees with their higher IMF content when compared to BI pigs. On the other hand, the contribution of lipolytic genes in the regulation of this balance remains uncertain, while the higher leptin and adiponectin signalling in the obese AL suggest that these hormones fail to stimulate lipolytic pathways, possibly through post-transcriptional regulation. IB pigs, for example, are widely known for being naturally leptin resistant, with increased appetite and high potential for fattening and develop metabolic syndrome (Sanz-Fernandez et al., 2020).

Adiponectin, studied in our qPCR candidate gene panel, is known to activate the FA oxidation pathways, increasing glucose uptake from the blood to the muscle, boosting its energy supplies through AMPK (Yanai and Yoshida, 2019). Traditionally known as an adipokine exclusively expressed by the adipose tissue, more recently skeletal muscle has also been found as a source of adiponectin expression, with its several autocrine and paracrine functions being extensively studied (Krause et al., 2019). One of these examples is the ability for ADIPOQ to suppress NIK overexpression, which can become an issue and reduce insulin sensitivity over time (Choudhary et al., 2011). Thus, functional adiponectin can restore insulin sensitivity. However, our data suggest that the overexpression of ADIPOQ observed in AL pigs is an overcompensation mechanism because higher levels of this adipokine are generally associated with leaner individuals, while lower levels are detected in obese ones (Zhang et al., 2019). Consequently, we also suggest that AL pigs may have developed adiponectin resistance although further studies regarding the expression of adiponectin receptors *ADIPOR1* and *ADIPOR2* with a higher number of replicates, as well as a study of the circulating levels of adiponectin are needed to explore such hypothesis.

6.4. Joint analysis of the transcriptomic studies

When comparing the fat and muscle transcriptomic studies, one aspect stands out from the rest. The total DEG output, at an identical significance level (p<0.05), was much larger in the adipose tissue than in the skeletal muscle (458 vs. 49). This is particularly interesting since similar sequencing strategies were employed (standard depth of 30-60 million per sample) and approximately 97% of the reads were successfully mapped with the pig reference genome used, regardless of the tissue. A difference that may help explain these results is the fact that only 30% of the total number of genes in the pig genome were detected in the adipose tissue, while in LL that number rises to over 72%. Consequently, the impact of the 650 million reads in the scope of

30% of the coding pig genes was much more relevant in shaping our transcriptome results than the 760 million in the scope of 72%. Furthermore, the results suggest a lower relevance of skeletal muscle in influencing lipid content and composition through transcriptional and signalling regulation when compared to adipose tissue. Perception of adipose tissue as a mere energy store is outdated and its functions have been extended to a pivotal endocrine organ that secretes numerous substances that influence homeostasis and metabolism at a whole-body level. On the other hand, the combining transcriptomic interactions between skeletal muscle cells and adipocytes should have the most impact in defining fat and lean tissue depositions and their respective efficiency rates (Kokta et al., 2004). Finally, both studies found greater proportion of DEGs overexpressed in AL pigs, with 57% in DSF and 69% in the LL.

A total of four DEGs were found common to both transcriptome analysis. The overexpression of *CBX1* was observed in AL pigs in either DSF or LL (log2 FC = 0.954, $q<0.05$ and log2 FC = 1.300, $q<0.05$, respectively), supporting the importance of epigenetic mechanisms in regulating gene expression through histone modifications in this genotype. On the other hand, the overexpression of *INTS11* (log2 FC = −1.297, $q<0.01$ and log2 FC = −1.675, $q<0.01$) and *STMN3* (log2 FC = −2.058, $q<0.01$ and log2 FC = −4.033, $q<0.01$) were consistently found in BI pigs across both studies. Finally, *RUFY1* was found overexpressed in Bísaro's DSF and in Alentejano's LL tissue (log2 FC = −1.809, $q<0.01$ vs. log2 FC = 1.060, $q<0.05$, respectively). This suggests that different requirements are needed across these tissues concerning *RUFY1*'s contribution towards cellular homeostasis. While on the LL muscle *RUFY1* can be associated with the higher IMF content of AL pigs, in the DSF it is suggested that higher levels of glucose are being mobilized from the blood into BI's adipocytes for storage, suggesting that the higher fat stores in AL pigs are regulated through other mechanisms and lipogenesis start through other precursors. This could also mean that the insulin's role in promoting glucose uptake by the fat and muscle cells may differ. Further study of the insulin receptors at each target tissue may unveil their distinct insulin sensitivity progression which may help explain these findings (Pearson et al., 2016; Unnikrishnan, 2004).

Fat stores and IMF play important regulatory roles in either the adipose or skeletal muscle tissues. An oversupply of fat content in both tissues is strongly linked with decreased insulin sensitivity due to the obesity-induced chronic inflammatory state. This state increases the secretion of pro-inflammatory cytokines and influences the activation of several signalling pathways, including the non-canonical NF*k*B. Our work has associated the expression of *MAP3K14* in the muscle of AL pigs with the activation of this pathway, whereas in the adipose tissue the mitogen-activated kinase we found overexpressed in AL pigs was *MAP3K15* (log2 FC = 1.036, $q<0.05$). However, this kinase, also known as ASK3, has no previous description of activating such pathway and is usually associated with the regulation of apoptotic death triggered by cellular stresses (Kaji et al., 2010), which is connected to the previously mentioned higher adipocyte death signals in AL. The development of insulin resistance involves several complex biological mechanisms that are not yet fully understood, and despite BI's lower tendency to store fat when compared to AL pigs, that does not exclude the possibility of decreased insulin sensitivity to be occurring in these pigs.

Transcriptional profiling poses a challenge in its interpretation due to high tissue heterogeneity. In both studies, we observed that candidate genes related to lipogenesis were

consistently found overexpressed in AL pigs, agreeing with our measured phenotypical results, and their recognized fatter profile. However, this pattern was easier to identify in DSF. In LL, the RNA-seq technique did not provide enough data and a qPCR survey was carried out. Next-generation sequencing techniques such as RNA-seq provide a broad perspective, at a standard sequencing depth, over a tissue transcriptomics but still struggle with the analysis of low expression data, while differences within high read counts are more easily detected (Yang et al., 2013b). In our LL dataset, several key genes involved in lipid synthesis and regulation were found below the cut-off point regarding total read count in the filtering step, which determined their premature withdrawal of the differential expression analysis. For example, *FASN*, in the adipose tissue scored a great correlation coefficient between methods (0.92, $p<0.01$) while in the muscle, where it was excluded in the initial filtering (<50 reads per group), presented a much lower, barely significant correlation (0.58, $p<0.05$). Therefore, we suggest that genes with fewer reads detected through RNA-seq should be considered with care since they instigate more associated noise and differential expression estimations may be biased against low read count values (Esteve-Codina, 2018; Raithel et al., 2016). Besides the convenience of the RNA-seq method at a standard read depth (30–60 million reads) for a general overview of the transcriptomic universe, RT-qPCR may provide a more reliable source of information to assess low expressed genes, as our results suggest.

Throughout history, the main goals of pig breeding programs have been to improve phenotypical traits related to the amount of lean meat produced while reducing costs, such as growth rate, feed efficiency and carcass lean meat content. As a consequence, today's highly commercial lean breeds present hypertrophied muscle cells, with larger proportions of fast type 2B myofibers, conferring higher glycolytic capability with the cost of developing undesirable meat quality traits (Fazarinc et al., 2020). On the other hand, local pig breeds such as the AL and BI have not been extensively selected for these reasons or selected at all, presenting slower growth rates but a higher ability to store fat, providing superior meat quality features. Furthermore, these breeds are generally reared in outdoor production systems, taking advantage of the locally available natural resources. Evaluation of the genetic predisposition through comparative transcriptomic studies on obese animals and exploration of new molecular markers stand as current fields on animal research. This is because fat and its composition and content, directly influences meat and meat products quality and phenotypical traits usually present a high polygenic background. Furthermore, since the Budapest congress in 1984, the European Union recommended that each nation should defend and preserve their own local breeds, as a part of a cultural, scientific, zootechnical, economic and social responsibility (Oliveira et al., 2019). The relatively small population size of local pig breeds has not allowed a higher degree of genetic diversity when compared to selected genotypes, despite being scarcely selected in programs. This is because in close populations the new individuals will be more closely related to each other when compared to the previous generation which escalates the levels of inbreeding while reducing genetic diversity (Laval et al., 2000; Muñoz et al., 2019; Rubin et al., 2012).

The Alentejano pig is one of the local pig breeds with higher population in Portugal, provides base products for the manufacture of high-quality foods and is one of the economic pillars of the Alentejo region. On the other hand, Bísaro pigs play a similar role, particularly in the northern and central regions of Portugal. Despite their importance in local farms, the population

size of both these breeds is still menaced. Measures such as modern improvements in genetic programs are imperative to fight these tendencies, boost the competition levels of the national genetic resources and preserve their phenotypical traits, ensuring a future to our breeds.

Chapter 7

Conclusions and future perspectives

The transcriptome analysis of the AL and BI Portuguese local pig breeds in the DSF fat yielded a total of 458 DEGs (q<0.05) and contributed with valuable knowledge to what is metabolically occurring in both breeds. The overexpression of *ACLY*, *FASN* and *ME1*, suggest that the cascade of reactions that comprise the *de novo* lipid synthesis are upregulated in AL pigs and explain the significantly higher fat proportion, which helped to attain a higher carcass yield in these pigs when compared to BI. Furthermore, the overexpression of genes responsible for the elongation of very long chain FAs, such as *ELOVL6*, and for desaturation, such as *SCD,* also suggest a predominance of these processes in AL pigs. On the other hand, significantly higher levels of leptin in AL pigs suggest that this breed has developed leptin-resistance, as previously proposed for the genetically similar IB pig. Meanwhile, a set of genes that play important roles in growth, including *IGF2*, *FOS*, *FOSB* and others, were found significantly overexpressed in BI pigs. This supports the idea that development stimuli can occur and be regulated at a multiple tissue level. The functional analysis employed allowed us to propose that a higher lipolytic activity in BI pigs can contribute to explain the observed phenotypical results mediated by growth hormone and AMPK signalling. Cholesterol synthesis is also suggested to be favoured in AL pigs due to the activation of upstream regulators PPARG, SCAP and SREBF2. Furthermore, the higher recruitment of macrophages, granulocytes, and other immune cells in AL pigs, suggested by the detected biological functions by IPA, hint an increased adipocyte size allowing a higher capacity to store lipid molecules. The fact that CD40 signalling was one of the main canonical pathways detected and was found significantly activated in BI pigs, supports the idea that lower insulin sensitivity is mainly occurring in the AL breed due to the pro-inflammatory effect of the excessive amounts of lipids in the adipose tissue when compared to BI.

The transcriptome analysis of the *Longissimus lumborum* muscle only yielded a total of 49 DEGs. Our results successfully managed to associate genes coding for specific slow type myosin heavy chain components to each pig's breed, namely *MYH3* to BI and *MYH7* to AL. Both genes are molecular markers for slow type 1 fibres, and despite the phenotypical characterization suggesting a higher proportion of fast type fibres in BI, no markers for this type of fibres were found differently expressed between these breeds. On the other hand, the observed higher expression of *TNNT1* in AL pigs agrees with their lower values of shear force, suggesting the *rigor mortis* to occur to a less extent in this breed, causing less shortening of the myofibers, lowering the hardness of meat. Overexpression of *MAP3K14* in AL pigs may be associated with their lower loin proportion, induced insulin resistance, and increased inflammatory response via NFkB activation. Growth markers related to DNA methylation and histone modification were associated to BI (*SMYD5* and *LSP1*) while in AL these mechanisms suppress cell differentiation through other genes (*KDM2B* and *WDR91*), suggesting a solid influence of epigenetic mechanisms. Overexpression of *RUFY1* in AL pigs may explain the higher IMF content via higher GLUT4 recruitment and consequently higher glucose uptake that can be stored as fat. Several candidate genes for lipid metabolism tested by qPCR, excluded in the RNA-seq analysis due to low counts, such as *ACLY*, *ADIPOQ*, *ELOVL6*, *LEP* and *ME1*, were established to influence the observed differences in lipid deposition and composition between these breeds. The results agree with the fatter profile of the AL pig breed and adiponectin resistance in this genotype can be postulated as responsible for the overexpression of NIK, failing to restore insulin sensitivity.

In the future, functional and proteomic studies should be performed to confirm the preponderance of the candidate genes with the functions previously described, particularly ones that directly influence the processes of lipid deposition and composition and muscle development. This is because sometimes mRNA and protein expression levels struggle for good correlation scores since not all transcribed RNAs turn directly to protein. Besides controlling protein levels at the transcriptional level, the several post-transcriptional mechanisms that can occur before translation, grant the cell control over the expression of the mRNA at different timepoints of the cell cycle, providing the specific protein levels when required (Greenbaum et al., 2003). A polymorphism genotyping survey should also be employed to identify potential SNPs in the candidate genes and others that could amplify the desired phenotypical traits. These polymorphisms that may affect gene expression are usually present near the target gene itself (cis-regulatory elements) or their transcription starting locations. All this will allow the selection of better individuals for targeted breeding and improved generations since genome traits as well as gene expression levels can be hereditable (Frese et al., 2013).

Furthermore, histological studies in the DSF and skeletal muscle tissues should help to propose and establish a link between tissue composition and gene expression. In DSF, it is important to know the dynamics between adipocyte number and size and the proportion of each type. Our results suggest a higher number in BI (hyperplasia) but a higher cell size in AL pigs (hypertrophy), allowing more fat accumulation within each adipocyte which would agree with a higher proportion of white adipose cells.

In the LL, the balance between the percentage of each of the four myofiber types is essential to relate with the gene expression data. No markers for fast skeletal myofibers were found in our DEG dataset, which would suggest a similar proportion of both slow and fast fibre types between breeds, although different signalling mechanisms are proposed for each breed slow type muscle fibres. It would also be interesting to compare the fibre type proportion between our local breeds with those of modern genotypes such as Large White, Landrace and Duroc, particularly regarding the abundancy of fast glycolytic myofibers of which these breeds are known to present in great amounts (Klont et al., 1998).

Finally, additional blood studies should be carried out to confirm the suggested hypothesis that AL pigs have reduced insulin, adiponectin and leptin sensitivity and explore the possibility of a similar pattern in BI, although to a lower extent when compared to AL pigs.

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Appendix I - Chemical composition and physical properties characterizing the tissues *Longissimus lumborum*, *Semimembranosus*, *Gluteus medius* and dorsal subcutaneous fat of Alentejano (AL) and Bísaro (BI) pig breeds slaughtered at ~150kg BW (n=9 for each breed)

Trait	*Longissimus lumborum*			*Semimembranosus*			*Gluteus medius*			Dorsal subcutaneous fat		
	AL	BI	p-value	AL	BI	p-value	AL	BI	p-value	AL	BI	p-value
Water content (g/100 g)	71.0	72.1	< 0.01	73.7	73.7	NS	69.9	70.6	NS	5.10	5.80	NS
Total protein (g/100 g)	23.6	23.0	0.10	22.4	22.8	NS	21.7	22.4	NS	0.91	1.29	< 0.01
Total lipids (g/100 g)	-	-	-	-	-	-	-	-	-	88.9	83.7	< 0.01
Total intramuscular fat (g/100 g)	6.90	6.00	< 0.05	5.00	4.50	NS	9.00	6.20	< 0.01	-	-	-
Myoglobin content (mg/g)	0.86	0.45	< 0.01	1.93	1.75	NS	1.63	1.34	0.08	-	-	-
Total collagen (mg/g dry matter)	13.1	16.7	< 0.01	15.5	20.7	< 0.05	15.2	17.9	< 0.01	-	-	-
Soluble collagen (% total collagen)	10.9	9.4	NS	8.20	6.80	NS	8.50	8.80	NS	-	-	-
pH (24 h *post-mortem*)	5.72	5.49	< 0.01	5.76	5.51	< 0.05	5.62	5.58	NS	-	-	-
pH fall (45 min to 24 h)	0.91	0.72	< 0.01	0.55	0.65	NS	0.95	0.89	NS	-	-	-
Drip loss (g/100 g)	0.59	1.92	< 0.01	NA	NA	NA	NA	NA	NA	-	-	-
Thawing loss (%)	3.30	4.47	NS	NA	NA	NA	NA	NA	NA	-	-	-
Cooking loss (%)	25.4	26.2	NS	NA	NA	NA	NA	NA	NA	-	-	-
Warner-Bratzler shear force (N)	41.9	51.4	0.05	NA	NA	NA	NA	NA	NA	-	-	-
Lightness (CIE *L**)	45.1	50.0	< 0.01	35.4	38.4	0.05	39.2	40.3	NS	79.3	79.1	NS
Redness (CIE *a**)	11.5	10.9	NS	14.5	14.3	NS	12.3	12.2	NS	2.25	3.36	< 0.01
Yellowness (CIE *b**)	3.18	3.50	NS	6.70	8.00	0.07	5.50	5.80	NS	4.47	4.91	NS
Chroma (*C**)	11.9	11.5	NS	16.0	16.5	NS	13.4	13.6	NS	5.00	6.00	< 0.05
Hue angle (*H*°)	15.7	18.1	0.07	24.6	29.1	< 0.05	23.9	25.1	NS	63.3	56.4	< 0.05
Saturation	0.27	0.23	0.07	0.46	0.43	NS	0.34	0.33	NS	0.06	0.08	< 0.05

NA = Not available; NS = Not statistically significant; CIE = Commission internationale de l'éclairage.

Appendix II - Complete list of DEG's (q<0.05) in DSF from AL and BI pigs slaughtered at ~150 kg BW (n=4 for each breed)

Ensembl ID	Gene	log2 FC	padj
ENSSSCG00000036109	*BIRC7*	4.863	1.620E-27
ENSSSCG00000010131	*TXNRD2*	3.058	4.120E-10
ENSSSCG00000015027	*C11orf1*	2.680	1.160E-07
ENSSSCG00000010133	*ARVCF*	2.225	1.130E-06
ENSSSCG00000031616	*FOSB*	-2.028	1.130E-06
ENSSSCG00000013758	*ZSWIM4*	-1.956	1.690E-06
ENSSSCG00000039357		2.050	1.740E-06
ENSSSCG00000000493	*FRS2*	4.439	1.820E-06
ENSSSCG00000033685	*NOXA1*	2.183	1.990E-06
ENSSSCG00000004454	*ME1*	2.090	8.420E-06
ENSSSCG00000004832	*UBE3A*	1.999	9.870E-06
ENSSSCG00000035308		-1.591	9.870E-06
ENSSSCG00000022948	*SLC7A9*	-2.324	1.220E-05
ENSSSCG00000031003		2.241	2.350E-05
ENSSSCG00000000660	*A2M*	3.521	2.680E-05
ENSSSCG00000000767	*ATP6V1E1*	-2.005	3.470E-05
ENSSSCG00000005951	*TMEM71*	2.517	5.780E-05
ENSSSCG00000036724	*CRYAB*	1.457	5.860E-05
ENSSSCG00000007287	*GSS*	1.863	6.250E-05
ENSSSCG00000015549	*RNASEL*	-2.071	6.920E-05
ENSSSCG00000038457		2.673	2.508E-04
ENSSSCG00000021220	*CKB*	2.193	2.533E-04
ENSSSCG00000014117	*THBS4*	2.270	2.573E-04
ENSSSCG00000011500	*KBTBD8*	-2.987	3.002E-04

ENSSSCG00000009369	*COG6*	-1.372	3.616E-04
ENSSSCG00000036844	*GPR171*	2.310	3.616E-04
ENSSSCG00000037660		1.248	3.882E-04
ENSSSCG00000027169	*ENOSF1*	1.863	3.972E-04
ENSSSCG00000010494	*SORBS1*	1.629	4.053E-04
ENSSSCG00000039360	*LRFN4*	-1.805	4.148E-04
ENSSSCG00000012693	*CT55*	1.777	4.231E-04
ENSSSCG00000017359	*RUNDC3A*	1.277	4.649E-04
ENSSSCG00000012062	*TTC3*	1.375	4.680E-04
ENSSSCG00000001984	*KHNYN*	1.106	4.884E-04
ENSSSCG00000039104		-1.714	4.884E-04
ENSSSCG00000007998	*RHBDL1*	0.951	4.929E-04
ENSSSCG00000009793	*CLIP1*	1.274	4.945E-04
ENSSSCG00000035619	*TMEM135*	1.555	4.945E-04
ENSSSCG00000030681	*MYBPH*	1.192	0.001
ENSSSCG00000034388	*CYP8B1*	-2.240	0.001
ENSSSCG00000016078	*HSPE1*	2.165	0.001
ENSSSCG00000010105	*SMPD4*	-1.526	0.001
ENSSSCG00000008261	*HK2*	1.722	0.001
ENSSSCG00000032195		1.324	0.001
ENSSSCG00000035924	*SHISA7*	-1.426	0.001
ENSSSCG00000010854	*TMEM63A*	1.582	0.001
ENSSSCG00000017141	*AATK*	-1.773	0.001
ENSSSCG00000006983	*CNOT7*	1.808	0.001
ENSSSCG00000032536	*B3GNT8*	-1.977	0.001
ENSSSCG00000005753	*CAMSAP1*	-2.088	0.001
ENSSSCG00000033095	*KIN*	1.228	0.001
ENSSSCG00000035904	*RPL7A*	0.920	0.001
ENSSSCG00000031912		-0.999	0.001
ENSSSCG00000012825	*IKBKG*	1.064	0.001

ENSSSCG00000014023	*RUFY1*	-1.809	0.001
ENSSSCG00000028249	*SKA3*	1.105	0.001
ENSSSCG00000016991	*DUSP1*	-1.539	0.001
ENSSSCG00000008021	*TMEM204*	-1.856	0.001
ENSSSCG00000036810	*ARL11*	1.594	0.001
ENSSSCG00000011848	*TFRC*	1.165	0.001
ENSSSCG00000002457	*ITPK1*	1.944	0.001
ENSSSCG00000036201	*NPR3*	1.881	0.001
ENSSSCG00000033946	*DIDO1*	-1.367	0.001
ENSSSCG00000031925	*SHANK3*	-1.592	0.001
ENSSSCG00000014567	*TRIM66*	-1.874	0.001
ENSSSCG00000032986	*AKAP17A*	-1.097	0.001
ENSSSCG00000029291	*PIGZ*	-1.716	0.002
ENSSSCG00000013227	*KBTBD4*	1.062	0.002
ENSSSCG00000029944	*FASN*	1.691	0.002
ENSSSCG00000014038	*FAM193B*	-1.692	0.002
ENSSSCG00000033822	*THRSP*	1.495	0.002
ENSSSCG00000022725	*FLOT1*	0.973	0.002
ENSSSCG00000032841		-2.186	0.002
ENSSSCG00000038911	*KLF2*	-1.236	0.002
ENSSSCG00000026994	*ADAMTS10*	-1.250	0.002
ENSSSCG00000013737	*RNASEH2A*	1.119	0.003
ENSSSCG00000016863	*OXCT1*	1.212	0.003
ENSSSCG00000029403	*C16orf74*	1.069	0.003
ENSSSCG00000016433		-1.813	0.003
ENSSSCG00000033302		1.064	0.003
ENSSSCG00000000050	*NAGA*	1.032	0.003
ENSSSCG00000029573	*NASP*	1.738	0.003
ENSSSCG00000001757	*WDR61*	1.364	0.003
ENSSSCG00000011831	*APOD*	-1.175	0.003

ENSSSCG00000007430	*NEURL2*	1.060	0.003
ENSSSCG00000014632	*FAM160A2*	-1.380	0.003
ENSSSCG00000039342	*DNAI1*	1.178	0.003
ENSSSCG00000038405	*STMN3*	-2.058	0.003
ENSSSCG00000029602	*WDR37*	1.401	0.003
ENSSSCG00000003339	*INTS11*	-1.297	0.003
ENSSSCG00000002447	*CPSF2*	1.175	0.003
ENSSSCG00000040681	*FABP4*	1.061	0.004
ENSSSCG00000014288	*GDF9*	1.318	0.004
ENSSSCG00000004103	*PCMT1*	1.368	0.004
ENSSSCG00000023599	*TIMM8B*	1.050	0.004
ENSSSCG00000007970	*POLR3K*	0.989	0.004
ENSSSCG00000036797		-2.032	0.004
ENSSSCG00000015430	*RINT1*	1.392	0.004
ENSSSCG00000002354	*COQ6*	1.821	0.004
ENSSSCG00000000807	*SLC38A1*	1.775	0.005
ENSSSCG00000004698	*SERINC4*	1.231	0.005
ENSSSCG00000006365	*DEDD*	1.339	0.005
ENSSSCG00000003601	*HCRTR1*	0.921	0.005
ENSSSCG00000013598	*KANK3*	-1.169	0.005
ENSSSCG00000017421	*ACLY*	1.853	0.005
ENSSSCG00000001001		1.074	0.005
ENSSSCG00000011377	*LAMB2*	-1.056	0.005
ENSSSCG00000006219	*MTFR1*	1.652	0.005
ENSSSCG00000011561	*PRRT3*	0.884	0.005
ENSSSCG00000009826	*TCTN1*	1.178	0.005
ENSSSCG00000040793	*CTSD*	0.907	0.005
ENSSSCG00000025560	*PGLYRP2*	2.604	0.006
ENSSSCG00000013839	*RASAL3*	1.719	0.006
ENSSSCG00000016208	*ZFAND2B*	1.070	0.006

ENSSSCG00000038459	*SURF2*	0.748	0.006
ENSSSCG00000003000	*ITPKC*	-2.500	0.006
ENSSSCG00000017311	*MAPT*	-1.557	0.006
ENSSSCG00000030155	*PHF10*	0.996	0.006
ENSSSCG00000027294	*CDV3*	-1.002	0.006
ENSSSCG00000003950	*TIE1*	-1.491	0.007
ENSSSCG00000017356	*FAM171A2*	0.884	0.007
ENSSSCG00000017926	*SLC16A13*	1.440	0.007
ENSSSCG00000009216	*SPP1*	1.838	0.008
ENSSSCG00000008006	*FBXL16*	1.320	0.008
ENSSSCG00000015044	*NKAPD1*	1.050	0.008
ENSSSCG00000040160	*MMP24*	0.928	0.008
ENSSSCG00000038202	*PCNT*	-1.582	0.008
ENSSSCG00000038189	*SMCR8*	-1.342	0.009
ENSSSCG00000001832	*ACAN*	1.436	0.009
ENSSSCG00000033608	*LOXL2*	1.175	0.009
ENSSSCG00000022999	*ZNF232*	-1.450	0.009
ENSSSCG00000030095	*ZBTB16*	-1.344	0.009
ENSSSCG00000029761		-0.807	0.009
ENSSSCG00000037791	*NKD2*	1.288	0.009
ENSSSCG00000025858	*ELN*	-1.743	0.009
ENSSSCG00000036236	*ELOVL6*	1.236	0.009
ENSSSCG00000017571	*EME1*	0.853	0.009
ENSSSCG00000007717	*METTL27*	-1.608	0.009
ENSSSCG00000007978	*LOC100737768*	-1.651	0.010
ENSSSCG00000017797	*SLC6A4*	-1.475	0.010
ENSSSCG00000004207		-1.507	0.010
ENSSSCG00000006539	*SHC1*	0.872	0.010
ENSSSCG00000017415	*TTC25*	1.593	0.010
ENSSSCG00000022204	*AGRN*	-1.660	0.010

ENSSSCG00000017876	*CYB5D2*	-1.355	0.010
ENSSSCG00000003919	*MUTYH*	1.157	0.010
ENSSSCG00000008238	*ELMOD3*	0.873	0.010
ENSSSCG00000002042	*OXA1L*	0.908	0.010
ENSSSCG00000018027	*TVP23B*	-1.798	0.010
ENSSSCG00000013592	*ELAVL1*	0.978	0.010
ENSSSCG00000027459	*RNF13*	1.405	0.010
ENSSSCG00000040513	*AQP3*	-1.403	0.010
ENSSSCG00000005828	*EGFL7*	0.897	0.010
ENSSSCG00000018039		-0.841	0.010
ENSSSCG00000031562	*MZF1*	-1.404	0.011
ENSSSCG00000001457	*SLA-DQB1*	1.090	0.011
ENSSSCG00000008703	*ADRA2C*	-1.418	0.011
ENSSSCG00000007577	*AP5Z1*	-1.371	0.011
ENSSSCG00000015444	*LAMB1*	1.020	0.011
ENSSSCG00000018014	*ZNF18*	1.352	0.011
ENSSSCG00000008002	*CIAO3*	1.196	0.011
ENSSSCG00000022849	*IL2RA*	-1.695	0.011
ENSSSCG00000017925	*SLC16A11*	1.248	0.011
ENSSSCG00000024389	*SIGIRR*	1.301	0.012
ENSSSCG00000014370	*TMCO6*	0.984	0.012
ENSSSCG00000008983	*CCDC158*	1.238	0.013
ENSSSCG00000032600	*PNOC*	-2.348	0.013
ENSSSCG00000030408	*DDX58*	0.867	0.014
ENSSSCG00000014802	*NUMA1*	-0.855	0.014
ENSSSCG00000000894	*CCDC38*	0.923	0.014
ENSSSCG00000028627	*CYP2G1P*	-1.649	0.014
ENSSSCG00000002029	*MYH7*	-2.397	0.014
ENSSSCG00000035176	*SEMA6B*	-1.154	0.014
ENSSSCG00000005582	*STRBP*	1.684	0.014

ENSSSCG00000035399		1.190	0.014
ENSSSCG00000013482		1.052	0.015
ENSSSCG00000026044	*FDFT1*	1.029	0.015
ENSSSCG00000002313	*SLC10A1*	-1.460	0.015
ENSSSCG00000038095		-1.360	0.015
ENSSSCG00000039524		1.348	0.015
ENSSSCG00000034089		-2.648	0.015
ENSSSCG00000007309	*RBM39*	0.871	0.016
ENSSSCG00000001764	*SH2D7*	0.912	0.016
ENSSSCG00000003967	*ZMYND12*	1.874	0.016
ENSSSCG00000011885	*FBXO40*	1.205	0.016
ENSSSCG00000037471	*LLCFC1*	-1.731	0.016
ENSSSCG00000003196	*PTOV1*	-0.822	0.016
ENSSSCG00000021837	*STYXL1*	0.833	0.017
ENSSSCG00000011411		1.176	0.017
ENSSSCG00000033189	*FAM107A*	-1.250	0.017
ENSSSCG00000005914	*SHARPIN*	0.839	0.017
ENSSSCG00000017793	*ANKRD13B*	-0.857	0.018
ENSSSCG00000002353	*FAM161B*	0.950	0.018
ENSSSCG00000013106	*PTGDR2*	0.857	0.018
ENSSSCG00000027457	*FBXO22*	1.050	0.018
ENSSSCG00000014336	*EGR1*	-1.172	0.018
ENSSSCG00000007927	*PPL*	-1.305	0.018
ENSSSCG00000033367	*PLEKHA2*	1.523	0.019
ENSSSCG00000035037	*SIK1*	-1.304	0.019
ENSSSCG00000003360	*WRAP73*	0.748	0.019
ENSSSCG00000012783	*IDH3G*	0.809	0.019
ENSSSCG00000005922	*SMPD5*	0.943	0.019
ENSSSCG00000028210	*NT5C*	-1.154	0.019
ENSSSCG00000007951	*CREBBP*	-1.182	0.019

ENSSSCG00000016548	*PODXL*	-1.319	0.020
ENSSSCG00000027676	*NOP56*	0.824	0.020
ENSSSCG00000015595	*ATF3*	-1.905	0.020
ENSSSCG00000005902	*FOXH1*	-1.137	0.020
ENSSSCG00000023377	*EMP3*	0.859	0.020
ENSSSCG00000030042	*SBNO2*	0.939	0.021
ENSSSCG00000029519	*CIAPIN1*	0.955	0.021
ENSSSCG00000014041	*MXD3*	0.895	0.021
ENSSSCG00000023936	*MYRFL*	-0.917	0.021
ENSSSCG00000003660	*PABPC4*	-1.358	0.021
ENSSSCG00000012895	*LOC100525027*	0.890	0.022
ENSSSCG00000003253	*MYADM*	-1.301	0.022
ENSSSCG00000005715	*PRRC2B*	-1.232	0.022
ENSSSCG00000004565	*CA12*	-1.757	0.022
ENSSSCG00000011264	*CSRNP1*	-1.774	0.022
ENSSSCG00000039915	*R3HCC1*	1.011	0.022
ENSSSCG00000023611	*TNXB*	-1.235	0.022
ENSSSCG00000016216	*TUBA4A*	0.801	0.022
ENSSSCG00000006982	*ZDHHC2*	1.912	0.022
ENSSSCG00000034338		-0.958	0.022
ENSSSCG00000032429	*RAX2*	0.759	0.022
ENSSSCG00000002993	*CYP2A19*	-1.782	0.022
ENSSSCG00000013145	*DTX4*	1.430	0.022
ENSSSCG00000025667	*FBXO2*	0.850	0.022
ENSSSCG00000011446	*NEK4*	0.908	0.022
ENSSSCG00000003155	*PPP1R15A*	-1.100	0.022
ENSSSCG00000035839	*TOP3A*	-1.162	0.022
ENSSSCG00000001501	*VPS52*	0.836	0.022
ENSSSCG00000006164	*WNT8B*	1.860	0.022
ENSSSCG00000032985	*GOT2*	1.405	0.023

ENSSSCG00000017706	*HEATR9*	-1.741	0.023
ENSSSCG00000036547	*METTL7B*	0.767	0.023
ENSSSCG00000005437	*KLF4*	-1.103	0.023
ENSSSCG00000005084	*PPM1A*	-1.306	0.023
ENSSSCG00000008030	*TSR3*	1.010	0.023
ENSSSCG00000012326	*RIBC1*	0.808	0.023
ENSSSCG00000002495	*SYNE3*	-1.292	0.023
ENSSSCG00000002550		1.269	0.023
ENSSSCG00000024549	*LYL1*	-1.841	0.024
ENSSSCG00000001027	*BMP6*	0.782	0.024
ENSSSCG00000006245	*SDR16C5*	1.606	0.024
ENSSSCG00000018081	*ATP6*	1.267	0.024
ENSSSCG00000032644	*HLX*	-1.028	0.024
ENSSSCG00000011401	*LSMEM2*	0.718	0.024
ENSSSCG00000007979	*LUC7L*	-1.095	0.024
ENSSSCG00000035082	*PHF5A*	0.805	0.024
ENSSSCG00000002515	*SLC25A47*	0.984	0.024
ENSSSCG00000005594	*WDR38*	1.142	0.024
ENSSSCG00000018094	*CYTB*	1.172	0.024
ENSSSCG00000023204	*AXIN1*	-1.113	0.025
ENSSSCG00000011453	*ITIH4*	-0.945	0.025
ENSSSCG00000001702	*SLC35B2*	0.818	0.025
ENSSSCG00000004762	*DNAJC17*	0.956	0.025
ENSSSCG00000036623		-1.243	0.025
ENSSSCG00000005599	*RABEPK*	1.113	0.026
ENSSSCG00000033404		0.833	0.026
ENSSSCG00000000263	*TNS2*	-0.954	0.026
ENSSSCG00000014892	*USP35*	-1.926	0.026
ENSSSCG00000017667	*PTRH2*	1.067	0.026
ENSSSCG00000010554	*SCD*	2.311	0.026

ENSSSCG00000011199	*TBC1D5*	0.931	0.026
ENSSSCG00000032490		0.941	0.026
ENSSSCG00000017529	*CBX1*	0.954	0.026
ENSSSCG00000006533	*ADAM15*	-1.173	0.026
ENSSSCG00000028567	*BTNL9*	-1.509	0.026
ENSSSCG00000017928	*C12H17orf49*	0.961	0.026
ENSSSCG00000004755	*DLL4*	-1.519	0.026
ENSSSCG00000035029	*NOVA2*	-1.914	0.026
ENSSSCG00000021408	*TKT*	0.855	0.026
ENSSSCG00000037016	*ID1*	-1.825	0.026
ENSSSCG00000036001	*RNF170*	1.676	0.026
ENSSSCG00000007426	*ACOT8*	0.785	0.027
ENSSSCG00000029985	*ARL10*	-1.717	0.027
ENSSSCG00000021954	*TAS2R39*	-4.608	0.027
ENSSSCG00000034462		-1.442	0.027
ENSSSCG00000004578	*ANXA2*	0.817	0.027
ENSSSCG00000007540	*CABLES2*	0.696	0.027
ENSSSCG00000008981	*FAM47E*	1.273	0.027
ENSSSCG00000002383	*FOS*	-1.087	0.027
ENSSSCG00000011618	*GATA2*	-1.120	0.027
ENSSSCG00000017066	*GEMIN5*	0.744	0.027
ENSSSCG00000035098	*MED4*	1.649	0.027
ENSSSCG00000025460	*SPSB2*	0.801	0.027
ENSSSCG00000038575		-1.435	0.027
ENSSSCG00000000587	*AEBP2*	-1.275	0.027
ENSSSCG00000007665	*IRS1*	1.013	0.027
ENSSSCG00000022975	*AMT*	-0.997	0.027
ENSSSCG00000027357	*CSTB*	0.970	0.027
ENSSSCG00000031710	*BRF1*	-1.305	0.027
ENSSSCG00000030603	*TATDN1*	0.928	0.027

ENSSSCG00000008009	*LMF1*	-0.882	0.028
ENSSSCG00000005322	*NPR2*	-0.776	0.028
ENSSSCG00000011214	*NGLY1*	1.031	0.028
ENSSSCG00000039605		0.926	0.028
ENSSSCG00000037181		0.965	0.028
ENSSSCG00000035891	*ADGRB1*	-2.509	0.028
ENSSSCG00000034611	*MEAF6*	-2.046	0.028
ENSSSCG00000015115	*CBL*	1.248	0.028
ENSSSCG00000037646	*DNAJA4*	0.995	0.028
ENSSSCG00000002866	*CEBPA*	0.974	0.029
ENSSSCG00000020735	*MAP3K15*	1.036	0.029
ENSSSCG00000013582	*XAB2*	-1.578	0.029
ENSSSCG00000017403	*STAT3*	-0.940	0.029
ENSSSCG00000014296	*VDAC1*	1.275	0.029
ENSSSCG00000012293	*PRICKLE3*	0.802	0.029
ENSSSCG00000038164	*RAI1*	0.825	0.029
ENSSSCG00000000117	*C22orf23*	0.927	0.029
ENSSSCG00000003352	*CDK11B*	-0.810	0.029
ENSSSCG00000013732	*MAST1*	1.156	0.030
ENSSSCG00000040929	*TPT1*	0.692	0.030
ENSSSCG00000033711		0.864	0.030
ENSSSCG00000011299	*CLEC3B*	-0.916	0.030
ENSSSCG00000037558	*YY1*	0.677	0.030
ENSSSCG00000036801	*C6orf132*	1.670	0.030
ENSSSCG00000000401	*GLS2*	0.752	0.030
ENSSSCG00000010083	*PPIL2*	0.860	0.030
ENSSSCG00000004414	*CD164*	1.580	0.031
ENSSSCG00000028592	*TM2D2*	2.331	0.031
ENSSSCG00000025523	*COL2A1*	-1.253	0.032
ENSSSCG00000018734	*MIR1282*	0.904	0.032

ENSSSCG00000009522	*PCCA*	1.129	0.032
ENSSSCG00000004551	*ZNF609*	-1.205	0.032
ENSSSCG00000027905	*RBM7*	0.963	0.033
ENSSSCG00000013083	*CPSF7*	0.925	0.033
ENSSSCG00000033020		0.964	0.033
ENSSSCG00000017262	*SLC16A6*	-1.742	0.033
ENSSSCG00000029633	*USP24*	-1.516	0.033
ENSSSCG00000021436	*DFFB*	-1.435	0.033
ENSSSCG00000008013	*IGFALS*	-1.308	0.033
ENSSSCG00000014203	*MCC*	-1.403	0.033
ENSSSCG00000015734	*IMP4*	0.877	0.034
ENSSSCG00000008000	*RPUSD1*	-1.518	0.034
ENSSSCG00000029189	*DCHS1*	-1.488	0.034
ENSSSCG00000038717	*FARS2*	0.851	0.034
ENSSSCG00000006105	*GEM*	-1.460	0.034
ENSSSCG00000000863	*SYCP3*	1.298	0.035
ENSSSCG00000012847	*TALDO1*	0.866	0.035
ENSSSCG00000039175	*MARCH1*	1.467	0.035
ENSSSCG00000029570	*PXDN*	-0.711	0.035
ENSSSCG00000029300	*ALKBH4*	0.767	0.036
ENSSSCG00000002675	*DNAAF1*	-1.593	0.036
ENSSSCG00000018086	*ND4L*	1.493	0.036
ENSSSCG00000039665		1.091	0.036
ENSSSCG00000035212	*KLF6*	-1.439	0.036
ENSSSCG00000018087	*ND4*	1.292	0.036
ENSSSCG00000002367	*ISCA2*	0.731	0.037
ENSSSCG00000009385	*SERPINE3*	1.212	0.037
ENSSSCG00000000968	*SBF1*	-0.574	0.037
ENSSSCG00000024563	*CEP83*	1.401	0.037
ENSSSCG00000040464	*LEP*	1.376	0.037

ENSSSCG00000007507	*PCK1*	-1.959	0.037
ENSSSCG00000011474	*PXK*	2.108	0.037
ENSSSCG00000017772	*SDF2*	-2.195	0.037
ENSSSCG00000039632		1.685	0.037
ENSSSCG00000032612	*MILR1*	-1.453	0.037
ENSSSCG00000022014	*DCDC2B*	-1.527	0.037
ENSSSCG00000006524	*THBS3*	-1.710	0.037
ENSSSCG00000033177	*CDC26*	0.994	0.038
ENSSSCG00000006790	*WDR77*	0.832	0.038
ENSSSCG00000005890	*ZNF7*	0.781	0.038
ENSSSCG00000002815	*ADGRG1*	-1.536	0.038
ENSSSCG00000022786	*MOGS*	0.756	0.038
ENSSSCG00000036659	*NIPSNAP2*	1.107	0.038
ENSSSCG00000012743	*MTMR1*	0.727	0.038
ENSSSCG00000018095		2.181	0.038
ENSSSCG00000038688	*PGLYRP1*	-0.700	0.038
ENSSSCG00000036441	*PMP2*	-1.794	0.038
ENSSSCG00000002523	*CDC42BPB*	-1.089	0.038
ENSSSCG00000011342	*DHX30*	0.677	0.038
ENSSSCG00000036963	*LRRC25*	0.972	0.039
ENSSSCG00000031719	*SEMA4B*	0.839	0.039
ENSSSCG00000037562	*SLC2A4RG*	-0.946	0.039
ENSSSCG00000031804		0.810	0.039
ENSSSCG00000029918	*CEPT1*	1.137	0.039
ENSSSCG00000035239		1.445	0.039
ENSSSCG00000037567		1.112	0.039
ENSSSCG00000009241	*COPS4*	1.379	0.040
ENSSSCG00000011040	*CACNB2*	-1.285	0.040
ENSSSCG00000003384	*DNAJC11*	-1.023	0.040
ENSSSCG00000007954	*SLX4*	-1.184	0.041

ENSSSCG00000013630	*DNM2*	0.594	0.041
ENSSSCG00000039198	*PCGF2*	0.803	0.041
ENSSSCG00000039094	*PLEC*	-1.017	0.042
ENSSSCG00000031288	*CLN5*	1.011	0.042
ENSSSCG00000017328	*ARHGAP27*	-1.441	0.042
ENSSSCG00000034755		0.827	0.042
ENSSSCG00000005477	*FKBP15*	0.904	0.042
ENSSSCG00000012843	*PIDD1*	-1.776	0.042
ENSSSCG00000017569	*CHAD*	0.650	0.042
ENSSSCG00000029334	*MYT1L*	-1.919	0.042
ENSSSCG00000003376	*PLEKHG5*	-1.164	0.042
ENSSSCG00000017197	*FBF1*	-1.496	0.043
ENSSSCG00000015810		1.140	0.043
ENSSSCG00000004064	*ARID1B*	-1.368	0.043
ENSSSCG00000023760	*CLEC14A*	-1.170	0.043
ENSSSCG00000003505	*DDOST*	-0.884	0.043
ENSSSCG00000000973	*SELENOO*	-0.752	0.043
ENSSSCG00000031657	*JUND*	-0.709	0.043
ENSSSCG00000004847	*MPHOSPH10*	1.001	0.043
ENSSSCG00000006481	*GPATCH4*	0.670	0.043
ENSSSCG00000014088	*IQGAP2*	-1.195	0.043
ENSSSCG00000005995	*MTBP*	0.925	0.043
ENSSSCG00000010399	*NCOA4*	0.958	0.043
ENSSSCG00000007521	*NELFCD*	0.938	0.043
ENSSSCG00000012884	*PPP6R3*	-1.593	0.043
ENSSSCG00000014305	*SAR1B*	1.086	0.043
ENSSSCG00000032320	*TCIM*	-1.132	0.043
ENSSSCG00000003154	*GYS1*	0.912	0.044
ENSSSCG00000037188	*PCP2*	0.710	0.044
ENSSSCG00000009627		-1.406	0.044

ENSSSCG00000013764	*RFX1*	-1.253	0.044
ENSSSCG00000038715	*FLCN*	-1.288	0.044
ENSSSCG00000017498	*PPP1R1B*	-1.075	0.044
ENSSSCG00000006823	*GNAT2*	1.146	0.045
ENSSSCG00000017983	*PER1*	-1.066	0.045
ENSSSCG00000030167	*SLC25A39*	0.971	0.045
ENSSSCG00000013021		-0.910	0.045
ENSSSCG00000023130	*ASCC1*	0.759	0.045
ENSSSCG00000032329		0.889	0.045
ENSSSCG00000029539	*RAB23*	0.994	0.045
ENSSSCG00000020835		-1.092	0.045
ENSSSCG00000035293	*IGF2*	-0.884	0.045
ENSSSCG00000035577		-1.657	0.045
ENSSSCG00000003478	*CROCC*	-1.361	0.046
ENSSSCG00000004048	*TCP1*	0.908	0.046
ENSSSCG00000017188	*ZACN*	1.055	0.046
ENSSSCG00000033655	*TUBB2B*	0.913	0.046
ENSSSCG00000001075	*DEK*	-0.803	0.047
ENSSSCG00000025092	*CDK4*	0.710	0.047
ENSSSCG00000013763	*DCAF15*	-0.638	0.048
ENSSSCG00000029094	*PIK3R4*	-1.588	0.048
ENSSSCG00000017712	*AP2B1*	0.984	0.048
ENSSSCG00000035561	*SLX1A*	-1.318	0.048
ENSSSCG00000038763	*CERK*	-1.647	0.048
ENSSSCG00000017591	*NME2*	1.059	0.048
ENSSSCG00000036261	*CROCC2*	-1.131	0.048
ENSSSCG00000030610	*FNBP4*	-1.237	0.048
ENSSSCG00000012992	*FRMD8*	-3.720	0.048
ENSSSCG00000017297	*CYB561*	-1.853	0.048
ENSSSCG00000007649	*GPC2*	1.226	0.048

ENSSSCG00000020526	*U5*	-1.599	0.048
ENSSSCG00000035999	*NUDT16L1*	-0.596	0.048
ENSSSCG00000023228	*NUMBL*	-1.116	0.048
ENSSSCG00000026686	*PDZD9*	0.710	0.048
ENSSSCG00000039541	*ANKRD11*	-0.915	0.049
ENSSSCG00000014634	*C11orf42*	-1.053	0.049
ENSSSCG00000013661	*EIF3G*	0.844	0.049
ENSSSCG00000013655	*ICAM1*	-1.839	0.049
ENSSSCG00000005620	*SH2D3C*	-1.348	0.049
ENSSSCG00000039466		-0.773	0.049
ENSSSCG00000017198	*ACOX1*	0.692	0.049
ENSSSCG00000027334	*EIF1AD*	0.662	0.050
ENSSSCG00000003428	*MTHFR*	-1.419	0.050
ENSSSCG00000008077	*CARD19*	-1.019	0.050

Appendix III - Complete list of DEG's (q<0.05) in LL from AL and BI pigs slaughtered at ~150 kg BW (n=5 for each breed)

Ensembl ID	Genes	log2 FC	padj
ENSSSCG00000003339	*INTS11*	-1.675	6.107E-09
ENSSSCG00000038405	*STMN3*	-4.033	2.324E-05
ENSSSCG00000008201	*CNNM3*	4.487	1.892E-04
ENSSSCG00000014835	*C2CD3*	4.438	1.966E-04
ENSSSCG00000034014	*MRPL40*	-0.936	0.001
ENSSSCG00000016532	*WDR91*	2.818	0.001
ENSSSCG00000024071	*SCARF1*	3.270	0.001
ENSSSCG00000001463	*PSMB9*	1.595	0.001
ENSSSCG00000008305	*SMYD5*	-2.171	0.001
ENSSSCG00000036026	*SRARP*	3.006	0.001
ENSSSCG00000031487	*LSP1*	-1.052	0.001
ENSSSCG00000026297	*KLB*	0.786	0.002
ENSSSCG00000009808	*TMEM120B*	1.232	0.002
ENSSSCG00000013894	*IL12RB1*	2.371	0.004
ENSSSCG00000017330	*MAP3K14*	1.829	0.005
ENSSSCG00000026754	*ATCAY*	1.365	0.005
ENSSSCG00000026894	*NFE2L3*	-1.606	0.005
ENSSSCG00000036783	*CCDC127*	2.948	0.007
ENSSSCG00000008503	*FEZ2*	1.046	0.007
ENSSSCG00000037201	*MPV17*	1.356	0.007
ENSSSCG00000033597		-1.887	0.007
ENSSSCG00000023318	*MARS1*	1.677	0.011
ENSSSCG00000022655	*PTGES2*	1.551	0.011

ENSSSCG00000033456	*GPR157*	1.670	0.015
ENSSSCG00000032652	*SLFN11*	1.335	0.015
ENSSSCG00000035969	*THRA*	0.954	0.015
ENSSSCG00000035852	*CSNK1D*	-0.674	0.017
ENSSSCG00000011437	*ALAS1*	2.322	0.018
ENSSSCG00000001595	*DAAM2*	0.986	0.018
ENSSSCG00000017529	*CBX1*	1.300	0.018
ENSSSCG00000007422	*DNTTIP1*	2.650	0.018
ENSSSCG00000035067		1.558	0.018
ENSSSCG00000022955	*POLR2B*	0.721	0.018
ENSSSCG00000003569	*SLC9A1*	1.997	0.018
ENSSSCG00000018007	*MYH3*	-1.191	0.030
ENSSSCG00000014023	*RUFY1*	1.060	0.032
ENSSSCG00000039206		0.930	0.033
ENSSSCG00000039148	*P4HB*	1.171	0.033
ENSSSCG00000031201	*LMOD1*	-0.714	0.037
ENSSSCG00000029160		-1.849	0.037
ENSSSCG00000040253		2.394	0.037
ENSSSCG00000029803	*KDM2B*	1.074	0.037
ENSSSCG00000015588	*ANGEL2*	-1.883	0.038
ENSSSCG00000015566	*EDEM3*	-1.582	0.044
ENSSSCG00000006054	*ATP6V1C1*	0.760	0.046
ENSSSCG00000023437	*ITPR1*	0.953	0.048
ENSSSCG00000030425	*SPHK2*	1.370	0.048
ENSSSCG00000037843	*TMEM138*	-1.112	0.048
ENSSSCG00000013050	*MARK2*	-1.196	0.050

Appendix IV - Chemical composition, and CIE colour parameters from the subset of DSF samples from AL and BI pigs slaughtered at ~150 kg BW

	AL (n=4)		BI (n=4)		p-value
	Mean	SE	Mean	SE	
Moisture (g/100 g)	4.74	0.11	5.15	0.70	0.565
Total protein (g/100 g)	0.94	0.10	1.42	0.07	0.006
Total lipids (g/100 g)	88.5	0.6	82.8	1.5	0.012
Lightness (CIE *L)**	78.8	0.3	79.6	0.3	0.092
Redness (CIE *a)**	2.49	0.31	3.54	0.71	0.224
Yellowness (CIE *b)**	4.15	0.33	4.82	0.45	0.269
Chroma (C*)	4.84	0.44	6.02	0.74	0.218
Hue angle (H°)	59.2	1.3	55.3	4.4	0.422
Saturation	0.06	0.01	0.08	0.01	0.236

Appendix V - Major fatty acid profile[1] from the subset of DSF samples of AL and BI pigs slaughtered at ~150 kg BW

	AL (n=4)		BI (n=4)		Significance
	Mean	SE	Mean	SE	
C14:0	1.40	0.12	1.48	0.11	0.677
C16:0	23.97	0.46	25.37	1.05	0.268
C16:1 n-7	1.75	0.03	2.00	0.09	0.030
C18:0	13.93	0.42	13.98	0.52	0.946
C18:1 n-9	42.04	0.53	37.69	0.35	<0.001
C18:1 n-7	2.92	0.13	3.15	0.45	0.613
C18:2 n-6	8.64	0.38	11.24	0.77	0.024
C18:3 n-3	0.49	0.02	0.62	0.05	0.059
C20:1 n-9	1.38	0.10	0.98	0.07	0.015
C20:2 n-6	0.60	0.01	0.49	0.04	0.026
∑ SFA	40.17	0.78	41.54	1.06	0.340
∑ MUFA	49.38	0.63	45.43	0.40	0.002
∑ PUFA	10.45	0.45	13.03	0.88	0.041
UFA/SFA	1.49	0.05	1.41	0.06	0.338
PUFA/SFA	0.26	0.01	0.32	0.03	0.133
∑ n-3	0.74	0.04	0.79	0.06	0.471
∑ n-6	9.84	0.38	12.40	0.86	0.034
n-6/n-3	13.38	0.21	15.74	0.83	0.033
∑ n-9	43.89	0.57	39.23	0.47	<0.001
Saturation index	0.44	0.01	0.47	0.01	0.041
Atherogenic index	0.49	0.02	0.54	0.03	0.344
Thrombogenic index	8.80	0.27	10.45	0.54	0.034

Notes: [1]Fatty acids with proportions lower that 0.5% of the total fatty acids identified are not shown;
SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; UFA – Unsaturated fatty acids;
Saturation index = (C14:0 + C16:0 + C18:0)/(ΣMUFA + ΣPUFA);
Atherogenic index = [C12:0 + (4xC14:0) + C16:0]/(ΣMUFA + Σn6 + Σn3).

Appendix VI - Chemical composition, physical properties and CIE colour parameters from the subset of LL samples of AL and BI pigs slaughtered at ~150 kg BW

	AL (n=5)		BI (n=5)		p-value
	Mean	SE	Mean	SE	
Moisture (g/100 g)	70.6	0.2	72.3	0.5	0.008
Total protein (g/100 g)	23.7	0.4	23.4	0.3	0.561
Total intramuscular fat (g/100 g)	7.3	0.2	5.7	0.2	0.001
Myoglobin content (mg/g)	0.83	0.12	0.43	0.04	0.014
Total collagen (mg/g DM)	13.7	0.6	16.3	0.7	0.025
pH (24 h post-mortem)	5.76	0.03	5.50	0.04	0.001
Drip loss (g/100 g)	0.55	0.07	2.25	0.21	<0.0001
Thawing loss (g/100 g)	3.38	0.98	5.28	0.78	0.167
Cooking loss (g/100 g)	24.6	1.0	27.3	0.6	0.056
Warner-Bratzler shear force (N)	39.6	2.1	51.3	5.3	0.072
Lightness (CIE *L)**	44.8	1.0	49.6	0.6	0.003
Redness (CIE *a)**	11.4	0.7	9.7	0.7	0.147
Yellowness (CIE *b)**	3.09	0.11	3.32	0.14	0.226
Chroma (*C*)	11.8	0.7	10.3	0.7	0.172
Hue angle (*H°*)	15.3	1.0	19.1	1.1	0.035
Saturation	0.26	0.02	0.21	0.02	0.041

Appendix VII - Major fatty acid profile[1] from the subset of LL samples of AL and BI pigs slaughtered at ~150 kg BW

	AL (n=5)		BI (n=5)		Significance
	Mean	SE	Mean	SE	
C14:0	0.94	0.07	1.10	0.11	0.225
C16:0	22.28	0.48	21.22	0.44	0.139
C16:1 n-7	3.14	0.31	2.63	0.16	0.179
C18:0	10.77	0.42	12.38	0.23	0.009
C18:1 n-9	35.00	1.47	29.91	0.59	0.012
C18:1 n-7	5.82	0.16	5.61	0.08	0.266
C18:2 n-6	12.06	0.59	15.17	0.60	0.002
C20:1 n-9	0.76	0.04	0.61	0.09	0.140
C20:3 n-6	0.57	0.11	0.75	0.05	0.142
C20:4 n-6	4.80	0.72	6.00	0.49	0.203
C22:4 n-6	0.64	0.09	0.78	0.10	0.317
∑ SFA	34.68	0.86	35.24	0.48	0.587
∑ MUFA	45.42	1.98	39.47	0.46	0.019
∑ PUFA	19.90	1.45	25.29	0.40	0.007
UFA/SFA	1.89	0.07	1.84	0.04	0.539
PUFA/SFA	0.57	0.04	0.72	0.02	0.011
∑ n-3	1.03	0.13	1.32	0.06	0.071
∑ n-6	18.82	1.33	23.76	0.40	0.007
n-6/n-3	18.84	1.31	18.12	0.88	0.661
∑ n-9	35.52	1.46	30.68	0.64	0.016
Saturation index	0.52	0.02	0.54	0.02	0.544
Atherogenic index	0.40	0.01	0.40	0.01	0.900
Thrombogenic index	14.07	1.08	17.66	0.21	0.012

Notes: [1]Fatty acids with proportions lower that 0.5% of the total fatty acids identified are not shown;
SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; UFA – Unsaturated fatty acids;
Saturation index = (C14:0 + C16:0 + C18:0)/(ΣMUFA + ΣPUFA);
Atherogenic index = [C12:0 + (4xC14:0) + C16:0]/(ΣMUFA + Σn6 + Σn3);
Thrombogenic index = (C14:0 + C16:0 + C18:0)/[(0.5xΣMUFA) + (0.5xΣn6) + (3xΣn3) + (Σn3/Σn6)].