Universitat Autònoma de Barcelona

OMICS APPROACHES FOR OMEGA-6/OMEGA-3 POLYUNSATURATED FATTY ACID RATIO IN PIGS

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fan constar

que el treball de recerca i la redacció de la memòria de la tesi doctoral titulada "*Omic approaches for omega-6/omega-3 polyunsaturated fatty acid ratio in pigs*" han estat realitzats sota la seva direcció per

YRON JOSEPH YABUT MANAIG

i certifiquen

que aquest treball s'ha dut a terme al Departament de Ciència Animal i del Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, al Departament de Veterinària i Ciències Animals de la Facultat de Veterinària de la Universitat de Milà i a la unitat de Genòmica Animal del Centre de Recerca en Agrigenòmica,

i consideren

que la memòria resultant es apta per optar al grau de Doctor en Producció Animal per la Universitat Autònoma de Barcelona i de Doctor en Veterinària i Ciència Animal per la Universitat de Milà.

I perquè en quedi constància, signen aquest document a Bellaterra, a 2 de Novembre del 2022.

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Para sa aking nanay at tatay,
kay ate, kay ate kuy,
at sa aking buong pamilya,
ang aking buhay, sandalan at lakas.
Para sa aking gabay araw-araw,
taos-pusong pagmamahal at pasasalamat sa lahat.

“…malayong lupain, amin mang marating
di rin magbabago ang damdamin.”

– Nicanor Abelardo at Teogenes Velez

CONTENT

SUMMARY

In general, meat quality is heavily influenced by fatty acid (FA) composition and intramuscular fat, which is mostly dependent on the dietary fat intake of pigs, but it is also affected by genetic variants. In this PhD thesis, we elucidated the effects of dietary lipids, in terms of omega-6 (n-6)/omega-3 (n-3) polyunsaturated fatty acid (PUFA) ratio, on porcine meat quality and the integration of OMICS technologies such as genomics, transcriptomics, and proteomics.

With the aim of understanding the association between n-6/n-3 PUFA ratios, porcine transcriptome expression, and biological processes related to PUFA metabolism, we performed RNA-Seq and microRNA-Seq on *longissimus dorsi* muscle samples from 20 Iberian x Duroc pigs with extreme values for n-6/n-3 FA ratio and identified differentially expressed messenger RNAs (mRNAs) and microRNAs (miRNAs). Our findings highlighted mRNA genes, miRNAs and enriched pathways that were related to lipid metabolism, cell growth and inflammation, according to differences in muscle n-6/n-3 PUFA ratio. Relevant miRNA-to-mRNA regulatory networks were also uncovered (i.e., *mir-15b* to *ARRDC3*; *mir-7142-3p* to *METTL21C*), and linked to lipolysis, obesity, myogenesis, and protein catabolism.

We also determined on how sow diets with high or low n-6/n-3 PUFA ratios of 13:1 (SOY) and 4:1 (LIN), influenced the reproductive performance of sows, growth performance of pre-weaned piglets, and fat deposition, including proteins and protein-coding genes using proteomics analysis. In sows, LIN decreased dead-born piglets and pre-weaning mortality. Although piglet weight and weight gain varied in between n-6/n-3 PUFA ratios, weaning weight and overall weight gain were not affected by dietary interventions. Proteomic analysis revealed 4 and 11 overabundant proteins in muscle and adipose tissues, respectively, in SOY compared to LIN. Additionally, the pro-inflammatory role of n-6 PUFAs may be associated to the observed overabundance of haptoglobin, an acute-phase

protein, and the stimulation of protein-coding genes and proteins relevant to the innate immune response and acute inflammatory response.

Using the same population, we applied the use of exon-intron split analysis (EISA) approach to account for post-transcriptional changes, driven by putative functional miRNA repression, in response to extreme values of n-6/n-3 PUFA ratio in *longissimus dorsi* muscle in male and female piglets. EISA revealed 11 and 97 mRNA genes within the top 5% negative PTc scores with at least 1.5-fold exonic region reduction in SOY-male (SOY-M) and LIN-male (LIN-M) piglets, respectively. Furthermore, the detected upregulated miRNAs harbored binding sites on 72.73% (SOY-M) and 61.86% (LIN-M) of these post-transcriptionally downregulated genes. Moreover, the overall PTc signals in females were not as strong and clear as those from the males. In addition, we discovered genes that are predominantly associated in the modulation of immune responses and lipid-related metabolism, which may be related to the pro- and anti-inflammatory properties of n-6 and n-3 PUFAs, respectively. Our use of EISA enabled us to identify regulatory networks that complemented traditional analyses of differential expression, providing us a more comprehensive understanding of muscle metabolic changes in response to PUFA concentration.

RESUMEN

En general, la calidad de la carne está fuertemente influenciada por la composición de los ácidos grasos (AG) y la grasa intramuscular, que depende en gran medida de la ingesta de grasa en la dieta de los cerdos, pero también esta influenciada por variantes genéticas. En esta tesis doctoral, dilucidamos los efectos de los lípidos de la dieta, en términos de la proporción de ácidos grasos poliinsaturados (AGP) omega-6 (n-6)/omega-3 (n-3), sobre la calidad de la carne porcina y la integración de tecnologías OMICAS como la genómica, la transcriptómica y la proteómica.

Con el objetivo de entender cómo la la asociación entre los ratios n-6/n-3 AGP, la expresión del transcriptoma porcino y los procesos biológicos relacionados con el metabolismo de los AGP, realizamos *RNA-Seq* y *microRNA-Seq* en muestras de músculo *longissimus dorsi* de 20 cerdos Ibéricos x Duroc con valores extremos de la relación de los AGP n-6/n-3 e identificamos ARN mensajeros (ARNm) y microARNs (miARNs) expresados diferencialmente. Nuestros hallazgos destacaron genes de ARNm, miARNs y vías enriquecidas que estaban relacionados con el metabolismo de los lípidos, el crecimiento celular y la inflamación, de acuerdo con las diferencias en la proporción de AGP n-6/n-3 del músculo. Además, se descubrieron redes reguladoras relevantes de miARN a ARNm (por ejemplo, *mir-15b* a *ARRDC3*; *mir-7142-3p* a *METTL21C*), y se relacionaron con la lipólisis, la obesidad, la miogénesis y el catabolismo proteico.

También se determinó cómo las dietas para cerdas con proporciones altas o bajas de AGP n-6/n-3, de 13:1 (SOY) y 4:1 (LIN), influyeron en el rendimiento reproductivo de las cerdas, en el rendimiento del crecimiento de los lechones predestetados y en la deposición de grasa, incluyendo las proteínas y los genes codificadores de proteínas mediante el análisis proteómico. En las cerdas, LIN redujo los lechones nacidos muertos y la mortalidad predestete. Aunque el peso de los lechones y la ganancia de peso variaron entre las proporciones de AGP n-6/n-3, el peso al destete y la ganancia de peso global no se vieron

afectados por las intervenciones dietéticas. El análisis proteómico comparativo entre SOY y LIN, reveló 4 y 11 proteínas sobreabundantes en los tejidos muscular y adiposo, respectivamente. Además, el papel proinflamatorio de los AGP n-6 puede estar asociado a la sobreabundancia observada de haptoglobina, una proteína de fase aguda, y a la estimulación de genes codificadores de proteínas y proteínas relevantes para la respuesta inmune innata y la respuesta inflamatoria aguda.

Utilizando la misma población, aplicamos el enfoque de análisis de división de exón-intrón (EISA) para dar cuenta de los cambios post-transcripcionales, impulsados por la represión funcional putativa de miRNA, en respuesta a los valores extremos de la relación de AGP n-6/n-3 en el músculo *longissimus dorsi* en lechones machos y hembras. El EISA reveló 11 y 97 genes de ARNm dentro del 5% de las puntuaciones negativas del PTc con una reducción de la región exónica de al menos 1,5 veces en los lechones SOY-macho (SOY-M) y LIN-macho (LIN-M), respectivamente. Además, los miARNs regulados al alza detectados albergaban sitios de unión en el 72,73% (SOY-M) y en el 61,86% (LIN-M) de estos genes regulados a la baja de forma post-transcripcional. Sin embargo, las señales generales de PTc en las hembras no fueron tan fuertes y claras como las de los machos. También descubrimos genes que están predominantemente asociados en la modulación de las respuestas inmunitarias y el metabolismo de los lípidos, que pueden estar relacionados con las propiedades pro y antiinflamatorias de los AGP n-6 y n-3, respectivamente. Nuestro uso de EISA nos permitió identificar redes reguladoras que complementaron los análisis tradicionales de expresión diferencial, proporcionándonos una comprensión más completa de los cambios metabólicos del músculo en respuesta a la concentración de AGP.

SINTESI

In generale, la qualità della carne è fortemente influenzata dalla composizione degli acidi grassi (AG) e dal grasso intramuscolare, che dipende soprattutto dall'apporto di grassi nella dieta dei suini, ma è anche influenzata da varianti genetiche. In questa tesi di dottorato, abbiamo chiarito gli effetti dei lipidi alimentari, in termini di rapporto acidi grassi polinsaturi (PUFA) omega-6 (n-6)/omega-3 (n-3), sulla qualità della carne suina e l'integrazione di tecnologie OMICS come la genomica, la trascrittomica e la proteomica.

Per comprendere l'associazione tra i rapporti n-6/n-3 AGP, l'espressione del trascrittoma suino e i processi biologici correlati al metabolismo degli AGP, abbiamo eseguito *RNA-Seq* e *microRNA-Seq* in campioni di muscolo *longissimus dorsi* di 20 maiali iberici x Duroc con valori estremi di rapporto n-6/n-3 AGP e sono stati identificati RNA messaggeri (mRNA) e i microRNA (miRNA) differenzialmente espressi. I nostri risultati hanno evidenziato geni mRNA, miRNA e percorsi arricchiti correlati al metabolismo lipidico, alla crescita cellulare e all'infiammazione, in base alle differenze nel rapporto n-6/n-3 PUFA del muscolo. Inoltre, sono state scoperte reti di regolazione tra miRNA e mRNA (ad esempio, *mir-15b* e *ARRDC3*; *mir-7142-3p* e *METTL21C*) correlate alla lipolisi, all'obesità, alla miogenesi e al catabolismo proteico.

È stato inoltre determinato come le diete delle scrofe con rapporti n-6/n-3 PUFA alti o bassi, pari a 13:1 (SOY) e 4:1 (LIN), influenzino le prestazioni riproduttive delle scrofe, le prestazioni di crescita dei suinetti pre-svezzati e il deposito di grasso, comprese le proteine e i geni codificanti per le proteine mediante analisi proteomica. Nelle scrofe, il LIN ha ridotto i suinetti nati morti e la mortalità pre-svezzamento. Sebbene il peso e l'aumento di peso dei suinetti variassero a seconda dei rapporti n-6/n-3 PUFA, il peso allo svezzamento e l'aumento di peso complessivo non erano influenzati dagli interventi dietetici. L'analisi proteomica comparativa tra SOY e LIN ha rivelato 4 e 11 proteine sovrabbondanti rispettivamente nei tessuti muscolari e adiposi. Inoltre, il ruolo proinfiammatorio degli n-

6 PUFA può essere associato alla sovrabbondanza osservata di aptoglobina, una proteina della fase acuta, e alla stimolazione di geni e proteine codificanti per la risposta immunitaria innata e la risposta infiammatoria acuta.

Utilizzando la stessa popolazione, abbiamo applicato l'approccio dell'analisi exon-intron-split (EISA) per tenere conto dei cambiamenti post-trascrizionali, guidati dalla repressione funzionale putativa dei miRNA, in risposta a valori estremi del rapporto n-6/n-3 PUFA nel muscolo *longissimus dorsi* in suinetti maschi e femmine. L'EISA ha rivelato 11 e 97 geni mRNA entro il 5% dei punteggi PTc negativi con una riduzione della regione esonica di almeno 1,5 volte nei suinetti SOY-maschio (SOY-M) e LIN-maschio (LIN-M), rispettivamente. Inoltre, i miRNA up-regolati individuati ospitavano siti di legame nel 72,73% (SOY-M) e nel 61,86% (LIN-M) di questi geni post-trascrizionalmente down-regolati. Tuttavia, i segnali complessivi di PTc nelle femmine non erano così forti e chiari come quelli dei maschi. Abbiamo inoltre scoperto geni prevalentemente associati alla modulazione della risposta immunitaria e del metabolismo lipidico, che potrebbero essere correlati alle proprietà pro- e anti-infiammatorie dei PUFA n-6 e n-3, rispettivamente. L'uso dell'EISA ci ha permesso di identificare reti di regolazione che hanno integrato le tradizionali analisi di espressione differenziale, fornendoci una comprensione più completa dei cambiamenti metabolici nel muscolo in risposta alla concentrazione di PUFA.

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

PAPER I

PAPER II

LIST OF PUBLCATIONS

The present thesis is based on the work contained in the list of articles below:

- Paper I. **Manaig YJY**, Criado-Mesas L, Esteve-Codina A, Mármol-Sánchez E, Castelló A, Sánchez A, Folch JM. (2022). Identifying miRNA-mRNA regulatory networks on extreme n-6/n-3 polyunsaturated fatty acid ratio expression profiles in porcine skeletal muscle. *PLOS ONE*. Under review

- Paper II. **Manaig YJY**, Sandrini S, Panseri S, Tedeschi G, Folch JM, Sànchez A, Savoini G, Agazzi A. (2022). Low n-6/n-3 gestation and lactation diets influence early performance, muscle and adipose polyunsaturated fatty acid content and deposition, and relative abundance of proteins in suckling piglets. *Molecules* 27:2925. https://doi.org/10.3390/molecules27092925

- Paper III. **Manaig YJY**, Mármol-Sánchez E, Castelló A, Esteve-Codina A, Sandrini S, Savoini G, Agazzi A, Sánchez A, Folch JM. (2022). Exon-intron split analysis reveals post-transcriptional regulatory signals induced by high and low n-6/n-3 polyunsaturated fatty acid ratio diets in piglets. (Manuscript in preparation)

ABBREVIATIONS

AA	Arachidonic acid
ACACA	Acetyl-CoA Carboxylase Alpha
ACLS4	Acyl-CoA Synthetase Long Chain Family Member 4
ACSL	Long-chain acyl-CoA synthetase
ADG	Average daily gain
ADIPOQ	Adiponectin, C1Q And Collagen Domain Containing
ALB	Albumin
AMPK	Adenosine 5'-monophosphate-activated protein kinase
ARNTL	Aryl hydrocarbon receptor nuclear translocator like
ARRDC2	Arrestin domain containing 2
ARRDC3	Arresting domain containing 3
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BLUP	Best linear unbiased prediction
C18:2n-6	Linoleic acid
C18:3n-3	Alpha-linoleic acid
CA3	Carbonic anhydrase 3
CD	Cluster of differentiation molecules
CDHR1	Cadherin related family member 1
CLA	Conjugated linoleic acid
CNV	Copy variation number
COL22A1	Collagen type XXII alpha 1 chain
CPT1	Carnitine palmitoyltransferase 1
CYP2E1	Cytochrome P450 family 2 subfamily E member 1
CYP3A22	Cytochrome P450 family 3 subfamily A member 22
ddNTPs	Dideoxynucleotides
DE	Differentially expressed
DEG	Differentially expressed genes

DEP	Differentially expressed proteins
DGCR8	DiGeorge syndrome critical region 8
DHA	Docosahexaenoic acid
DIAPH1	Diaphanous related formin 1
DNA	Deoxyribonucleic acid
EFA	Essential fatty acids
EISA	Exon-intron split analysis
EJD	European Joint Doctorate
ELOVL2	ELOVL fatty elongase 2
ELOVL6	ELOVL fatty elongase 6
ELOVL6	ELOVL fatty acid elongase 6
ENHO	Energy homeostasis associated
EPA	Eicosapentaenoic acid
ESR	Early Stage Researcher
EST	Expressed sequence taqs
EU	European Union
EVI2B	Ecotropic Viral Integration Site 2B
FA	Fatty acids
FABP3	Fatty acid binding protein 3
FABP4	Fatty acid binding protein 4
FABP5	Fatty acid binding protein 5
FAO	Food and Agriculture Organization of the United Nations
FASN	Fatty acid synthase
FCR	Feed conversion ratio
FOXN2	Forkhead box
GLA	Gamma γ-linoleic acid
GO	Gene ontology
GPR120	G protein-coupled receptor 120
GWAS	Genome-wide association studies

HAL	Halothane gene
IGF2	Insulin like growth factor 2
IL-6	Interleukin 6
IMF	Intramuscular fat
IV	Iodine value
LA	Linoleic acid
LCFA	Long-chain fatty acids
LEP	Leptin
LEPR	Leptin receptor
LIN	diet with n-6/n-3 ratio of 4:1
LIPG	Lipase G
LOH	Loss of heterozygosity
LPL	Lipoprotein lipase
LXRα	Liver X receptor α
MANNA	European Joint Doctorate in Molecular Animal Nutrition
MC4R	Melanocortin 4 receptor
MCFA	Medium-chain fatty acids
METTL21C	Methyltransferase-like 21C
miRISC	miRNA-induced silencing complex
miRNA	microRNA
miRNA-Seq	microRNA sequencing
mRNA	Messenger RNA
MS	Mass spectrometry
MSTN	Myostatin
MTTP	Microsomal triglyceride transfer protein
MUFA	Monounsaturated fatty acids
MYPN	Myopalladin
n-3	omega-3 PUFA
n-6	omega-6 PUFA
n-6/n-3	omega-6/omega-3 PUFA

NADPH	Nicotinamide adenine dinucleotide phosphate
NGS	Next-generation sequencing
NRC	National Research Council
OECD	Organisation for Economic Co-operation and Development
PCIT	Partial Correlation with Information Theory
PCK1	Phosphoenolpyruvate carboxykinase 1
PGK2	Phosphoglycerate kinase 2
PGM sequencing	Personal genome machine sequencing
PHKG1	Phosphorylase Kinase Catalytic Subunit Gamma 1
PIC	Pig Improvement Company
PIGMaP	Pig Genome Mapping Project
PIK3CD	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta
POU1F1	POU class 1 homebox 1
PPARGC1A	PPARG coactivator 1 alpha
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PRKAG3	Protein kinase AMP-activated non-catalytic subunit gamma 3
PSS	Porcine stress syndrome
PTc	Post-transcription
PUFA	Polyunsaturated fatty acids
QTL	Quantitative trail loci
QTLdb	Quantitative trail loci database
r	Pearson's pairwise correlation coefficient
RBP4	Retinol binding protein 4
RN$^-$	Rendement napole gene
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RT-PCR	Reverse transcription-polymerase chain reaction
RYR1	Ryanodine receptor 1
SAGE	Serial analysis of gene expression

SBNO1	Strawberry notch homolog 1
SCD	Stearoyl-CoA desaturase
SCFA	Short-chain fatty acids
SFA	Saturated fatty acids
SGSC	Swine Genome Sequencing Consortium
SNP	Single nucleotide polymorphism
SOY	diet with n-6/n-3 ratio of 13:1
SREBP	Sterol regulatory-element binding proteins
ST8SIA2	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2
TAGs	Triglycerides
TF	Transcription factor
TGS	Third generation sequencing
TMEM71	Transmembrane protein 71
TTN	Titin
US	United States
UTR	Untranslated region
VGLL2	Vestigial like family member 2
VLCFA	Very long-chain fatty acids
WHC	Water holding capacity
XPO5	exportin 5
ΔEx	Change in exonic region

GENERAL INTRODUCTION

Chapter 1

1.1. Current trend of pork meat production

Meat is considered as the main source of highly concentrated proteins for humans and it mostly includes poultry, pork, and beef meat. Around 336 million tons of meat were produced worldwide, and one-third of which accounts to pork. From 1961 to 2018, it was reported that the world production of pork increased fivefold, whereas in Europe, the production amount was doubled, as shown in Fig. 1. Countries like Spain and Italy are also presented below, for comparison.

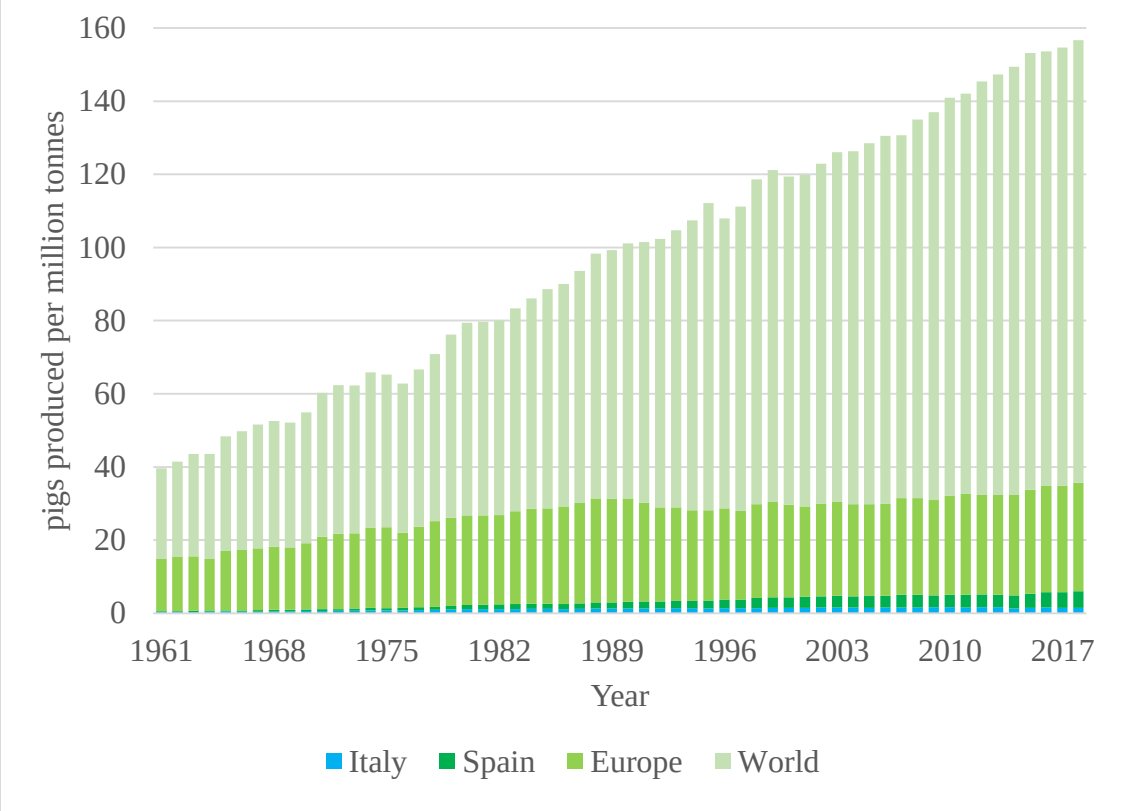

Figure 1. Pork meat production in the World, Europe, Spain, and Italy from 1961 to 2018, constructed based on the data on FAOSTAT (OECD/FAO, 2018).

The European Union is considered as the world's second biggest producer of meat after China, and the biggest exporter of pig meat and pig meat products. Nowadays, China, EU, and the United States compromise to almost 76% of the global pig production and 74% of its consumption. Pigs are one of the most important agricultural livestock animals

for meat production, amounting to a total of 122.5 million tons globally in 2021. FAO also highlighted that the recent expansion of world meat output was mainly driven by the increase in pork output (Food and Agriculture Organization of the United Nations (FAO), 2021). Pork consumption accounts to more than 40% of the total meat and poultry consumption shares around the world (OECD/FAO, 2018). Based on the OECD-FAO Agricultural Outlook of 2018, as the consumption level on developed countries becomes saturated, an increase of *per capita* consumption is projected on developing countries. The trend can be correlated to the increase in average individual incomes and to the growth of population at regional level. Moreover, OECD also forecasted that there will be stagnation from 2015 to 2025 on kg/capita/year on world's consumption of pork, beef, and veal. As presented in Fig. 2., EU ranks a close second after China, in terms of *per capita* pork consumption, which yields to 40.8 kg/capita/year. Consumption EU and US regions are growing slowly or even stagnating whereas in Asia and in other regions, an 80% growth forecast in the meat sector could be observed by 2022 (MEAT ATLAS, 2014).

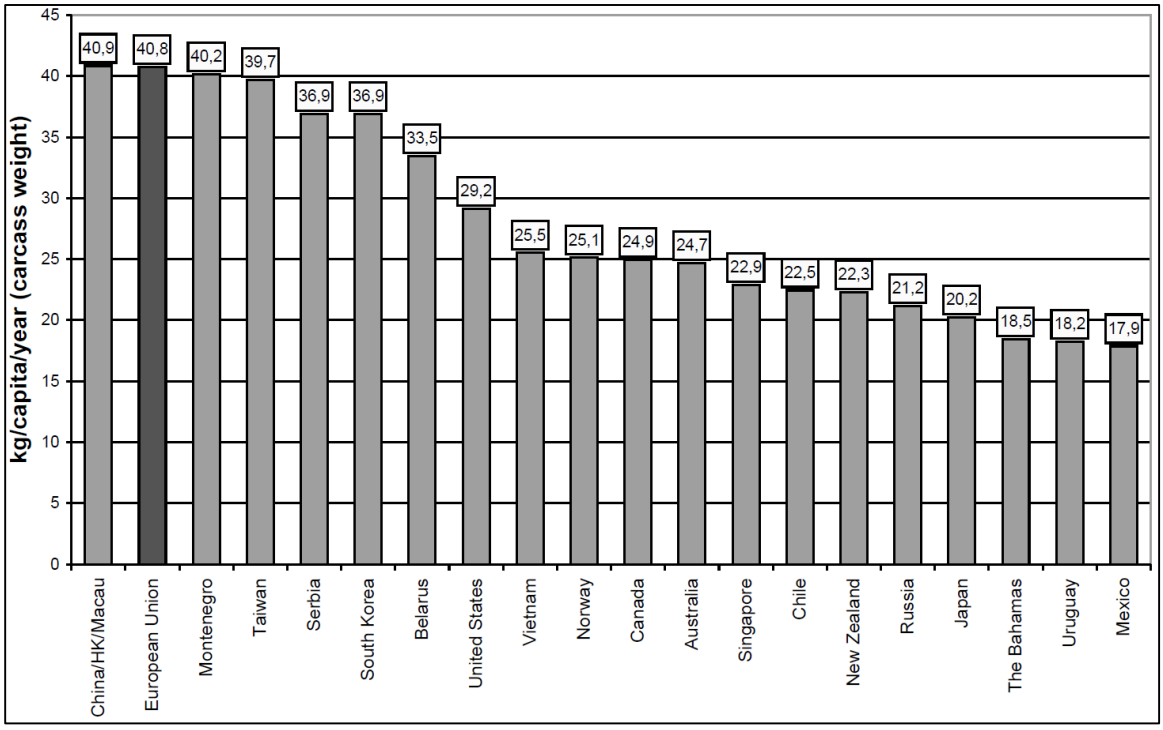

Figure 2. 2017 per capita pork consumption in the Top 20 countries in kilogram per capita per year of carcass weight (Szűcs & Vida, 2017).

In the past, increases in yield of livestock products were observed due to domestication and conventional livestock breeding techniques. At the same time, some considerable changes in the composition of livestock products have occurred; demands were attained due to combination of conventional techniques, cross-breeding, and within-breed selection, in which increases differently depending on regions (Thornton, 2010).

1.2. Pork meat quality

Pigs are raised to produce pork for consumers to eat. The improvement in the pork industry is mainly influenced by the pig breeding stock. This also determines its success or failure; it aims to provide superior genes to optimize the quality of pork for human consumption. Multiple factors can affect the pork quality – breed, genotype, feeding, pre-slaughter handling, stunning, slaughter method, chilling, and storage conditions. The interaction of these factors can determine and dictate the overall quality of pig meat. Genotype and nutrition affect fat content, composition, uniformity and oxidative stability while water-holding capacity and color are mostly affected by the factors listed above. Sensory qualities such as eating quality and appearance of the meat, are determined by tenderness, flavor, juiciness, and color. According to Pig Improvement Company (PIC), a 'high quality' meat cannot be solely defined as it is. It is a combination of subjective and objective measurements that varies across markets. Moreover, it is the consumer perception that drives the demand of what quality of meat should be produced as it is the main goal of the pork production. Studies have also shown that tenderness, juiciness, and flavor impose a high positive influence on the preference of the consumers (Aaslyng et al., 2007; Choi et al., 2014; Nold, 2006; Rosenvold & Andersen, 2003).

1.2.1. Intramuscular fat (IMF)

Increased meatiness and reduced fat content has now become the trend of the modern pork industry, mainly due to animal selection programs and improvements toward leanness. Such trend has caused the lack of tenderness, juiciness, and flavor of pork as it is

hard to sustain these qualities from lean pigs. Intramuscular fat (IMF) is defined as the fat within the muscle that is usually invisible at low levels but becomes highly visible at high levels, and generally referred to as 'marbling' (Sosnicki et al., 2010). Marbling affects tenderness, juiciness and flavor, all indicators of pork quality. It is also correlated to aroma volatiles and essential fatty acids. The lipid content of the meat has the most significant effect on its sensory properties (Brewer et al., 2001; Choi et al., 2014).

Quantification of IMF can be measured by analytical methods for isolation and purification of total lipids in animal tissues (Bligh & Dyer, 1959; Folch et al., 1957), or can be estimated *in vivo* by real-time ultrasound (Houghton & Turlington, 1992; Newcom et al., 2002), or by nuclear magnetic resonance techniques (Villé et al., 1997). The overall distribution of IMF varies among tissues. As per reported, the variation among the IMF composition between muscle and adipose tissues is around 25-30% (Segura et al., 2015). Sensory properties of pork are highly correlated to pork marbling. The flavors and juiciness of porcine meat are significantly enhanced when the IMF values are more than 2.5%; although, these values of IMF have to reach a minimum of 2.0% before the effects on sensory qualities could become noticeable (Bejerholm & Barton-Gade, 1986; Fernandez et al., 1999). On another hand, IMF content above 3.0% has a positive effect on the organoleptic properties of pork (i.e., palatability, juiciness, and tenderness) (Daszkiewicz et al., 2005).

1.2.2. Fatty acid composition

Fatty acid composition has been correlated to meat quality and its nutritional value. There has been an increasing interest on how to improve meat quality traits without affecting the production performance of the animal. Fats and oils are main stored forms of energy in animals. By definition, fatty acids (FA) are carboxylic acids with hydrocarbon chains ranging from 4 to 36 carbons long. They can be classified based on carbon chain length (Table 1) and their degree on saturation (number of double bonds present on its structure) (Table 2) (Nelson & Cox, 2008).

Table 1. Fatty acid classification based on carbon chain length.

Name	**Number of carbons**
Short-chain fatty acids (SCFA)	5 or fewer carbons
Medium-chain fatty acids (MCFA)	6 to 12 carbons
Long-chain fatty acids (LCFA)	13 to 21 carbons
Very long-chain fatty acids (VLCFA)	22 or more carbons

Moreover, FAs can also be classified as essential and nonessential. Nonessential FAs are fatty acids that can be synthesized by the animal whereas, essential FAs are those that cannot be synthesized by the body and must be obtained from the diet. Lipids can also be characterized as saponifiable or nonsaponifiable – production of fatty acid salts upon treatment with a base solution (Nelson & Cox, 2008).

Dietary fat consumed by the pigs directly affect the fatty acid composition of the carcass – it mimics the fatty acid composition of the diet (National Research Council, 2012). During digestion, dietary fatty acids are minimally hydrogenated or remain unchanged. If the pigs are fed above the maintenance requirement, efficiency of dietary fat utilization is 90%. Depending on the specific fatty acids, 31-40% of dietary fat is transferred to carcass lipid (Freeman, 1983; Kloareg et al., 2007). In response in dietary fat ingestion, *de novo* synthesis of fatty acids is inhibited to favor the direct deposition of fatty acids in adipose tissue (Chilliard, 1993; Farnworth & Kramer, 1987).

Table 2. Fatty acid classification based on degree of saturation.

Name	**Number of double bonds (C=C)**
Saturated fatty acids (SFA)	No double bonds
Monounsaturated fatty acids (MUFA)	One (C=C) double bond
Polyunsaturated fatty acids (PUFA)	Two or more (C=C) double bonds

The differences in fatty acid content on meat have effects on its quality – it regulates the firmness or oiliness of adipose tissue, oxidative stability of muscle, and both of their flavor and color. The current standard of fat firmness is the Iodine Value (IV). Determination of IV states the unsaturation of fats by the number of double bonds found

in fatty acids. It is usually expressed in terms of the absorption of iodine by a fat. Saturated fat has low iodine value which yields firmer fat whereas unsaturated fat, having double bonds, has high iodine value, hence, softer fat (Sosnicki et al., 2010). Issues with soft pork arises on bacon processing which makes them difficult to slice. Furthermore, it may lead to increased incidence of fat smear and fat separation (i.e., yellowing, wet, and oily appearance when packaged), and reduced shelf life since unsaturated fatty acids are susceptible to lipid oxidation (Norwood, 2013). Nowadays, nutritional intervention is being made to address the effects of the diet and FA composition on human health. Diets high in SFA are proven to contribute to the increase of low density lipoprotein-cholesterol level, which has positive correlation to cardiovascular diseases. MUFA and PUFA, on the other hand, have favorable effects on human health (Nieto & Ros, 2012).

1.3. Fatty acid metabolism

Lipids are a group of heterogeneous biomolecules that are insoluble in water and soluble in non-polar solvents. They serve as an energy source, insulation for vital organs, covering of nerve fiber, components of cell membranes, and chemical messengers in hormones. This biomolecule also constitutes lipoproteins, which enables the transport of lipids in aqueous environment and throughout the body. Saponifiable lipids includes triglycerides, glycerophospholipids, waxes, and glycolipids, whereas nonsaponifiable lipids comprises of steroids (i.e., cholesterol, bile salts, and hormones), eicosanoids, terpenes, pheromones, and fat-soluble vitamins (i.e., A, D, E, and K). Essential fatty acids like omega-3 and omega-6 are known to be precursors for eicosanoids (i.e., prostaglandins, thromboxanes, leukotrienes), which play an important role in pain, fever, inflammation, and blood clotting (Nelson & Cox, 2008; Stryer et al., 2002).

The biosynthesis of FA is known as lipogenesis, while its breakdown of such is called lipolysis. Lipogenesis or *de novo* FA synthesis happens when there is excessive caloric intake which stimulates the accumulation of fat in the adipose tissues as an energy source (Saponaro et al., 2015) whereas, lipolysis or β-oxidation occurs during fasting or

exercise. It involves the mitochondrial oxidation of triglycerides into FAs to provide energy for the cells (Duncan et al., 2007).

1.3.1. Fatty acid synthesis

The biosynthesis of fatty acids occurs in all vertebrate species. It was reported that through a number of metabolic stages, FA and triglycerides (TAGs) are synthesized generally through lipogenesis which occurs in intestinal mucosal cells, in liver, in adipose tissue, and in the mammary gland (i.e., lactating animals). FA can be obtained through diet or can be synthesized *de novo*. Excess carbohydrates mean excess acetyl-CoA – the surplus of this molecule cannot stay in the blood hence, it will be converted into FAs, and later, into TAGs. FAs are stored in adipose tissues as TAGs (Laliotis et al., 2010). The biosynthesis (Fig. 3) occurs in the cytosol, which requires acetyl-CoA, a 3-carbon intermediate (i.e., malonyl CoA), NADPH, and fatty acid synthase (FASN) complex – a multifunctional enzyme complex that has seven (7) enzymatic activities that catalyzes each step in fatty acid biosynthesis. The carboxylation of acetyl-CoA to malonyl-CoA is the committed step in this anabolism – this means that the synthesis of fatty acid will take place, irrevocably, and eventually end up with the pathway's final product (Stryer et al., 2002). The FASN system in animal cells produce palmitate as its principal product, in which can either undergo elongation or saturation by further addition of acetyl groups to its carbon chain. Long-chain SFA and MUFAs are then synthesized from the process (Fig. 4). Mammals lack the enzyme to incorporate double bond beyond carbon 9, hence, the necessity to obtain essential fatty acids, linoleic C18:2n-6, or α-linolenic C18:3n-3, from the diet. Arachidonic is one of the main components of membrane lipids. Along with omega-3 and omega-6 PUFAs, they serve as a precursor for the synthesis of eicosanoids such as prostaglandins, thromboxanes, leukotrienes, and lipoxins (Pelley, 2011).

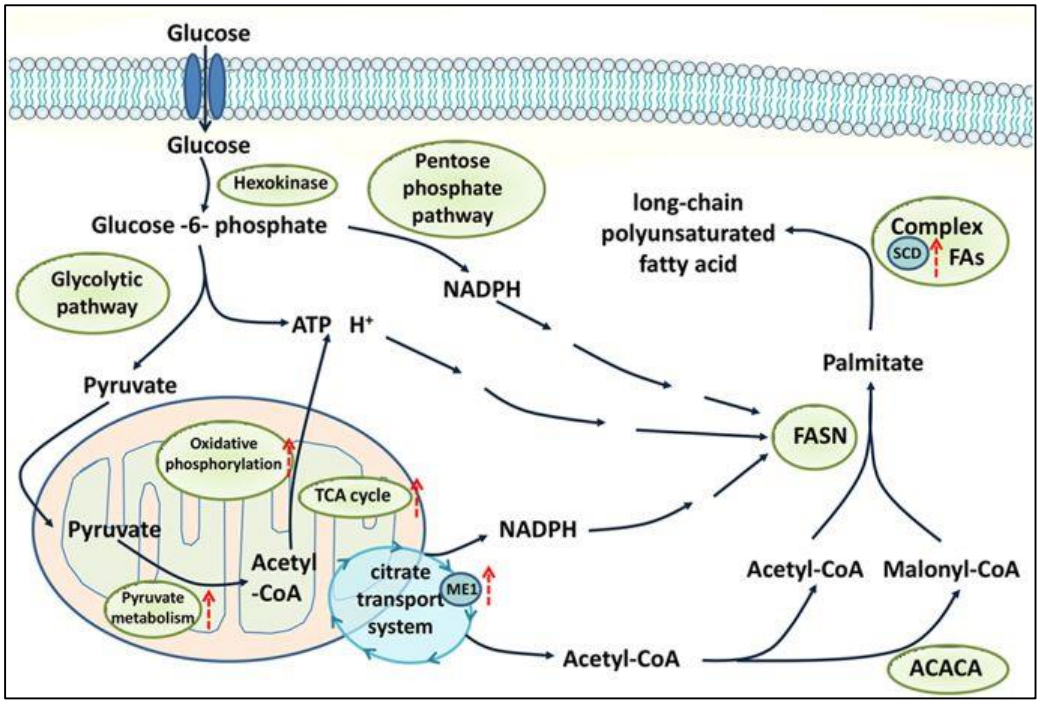

Figure 3. Overview of de novo fatty acid synthesis (Stryer et al., 2002).

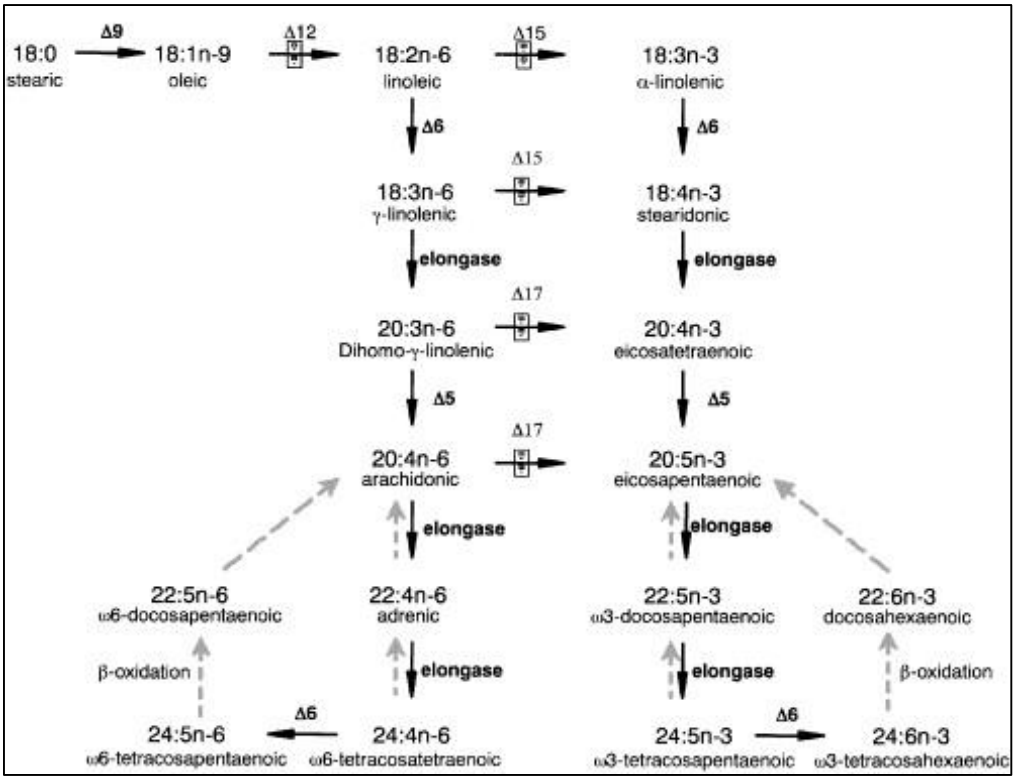

Figure 4. Synthesis of long chain fatty acids in mammals (Pereira et al., 2003).

1.3.2. Fatty acid oxidation

Digestion of lipids starts from the small intestine where the presence of TAGs triggers the secretion of cholecystokinin which prompts the release of pancreatic lipase and bile stored in the gall bladder. During fasting, stored energy in the form of TAGs will be oxidized due to the absence of glucose in the system (Stryer et al., 2002). The catabolism of FA occurs in three stages that occurs in mitochondria – (1) mobilization of lipid from adipose tissues to energy-requiring tissues, (2) FA activation and transport, (3) and β-oxidation of fatty acyl-CoA. Fatty acyl-CoA converts each 2-carbon unit of fatty acids into acetyl CoA and further oxidized to CO_2 during citric acid cycle (Nelson & Cox, 2008). During the process, electrons that are accumulated from the oxidation will be passed to O_2 via the mitochondrial respiratory chain, to provide the energy for ATP synthesis by oxidative phosphorylation (Lodish et al., 2000).

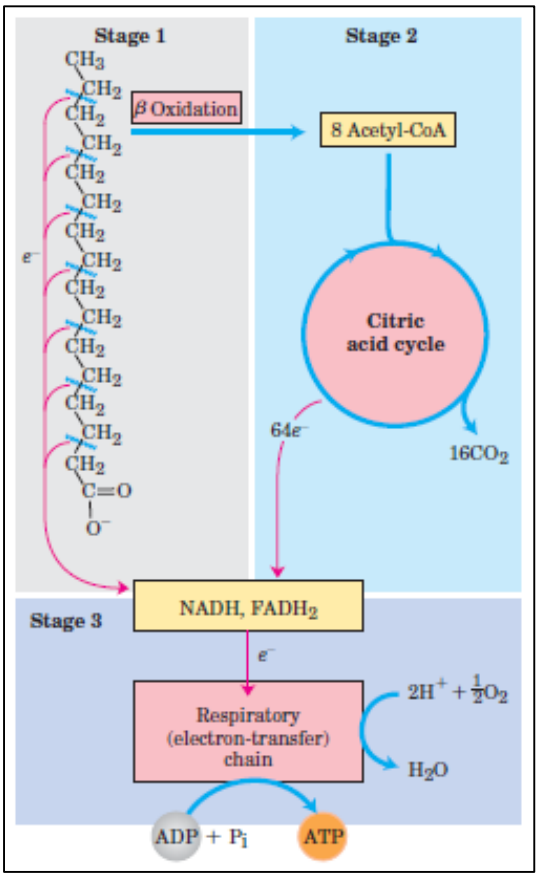

Figure 5. Fatty acid oxidation (Nelson & Cox, 2008).

1.4. OMICS Technologies

'Omic' is defined as the collective technologies primarily aimed at the detection of genes (genomics), RNA transcripts (transcriptomics), proteins (proteomics), and metabolites (metabolomics) in a non-targeted and non-biased method (Fig.6). They are used to explore the roles, relationships, and actions of the aforementioned that make up a cell, tissue, or organism (Horgan & Kenny, 2011; Ward, 2014).

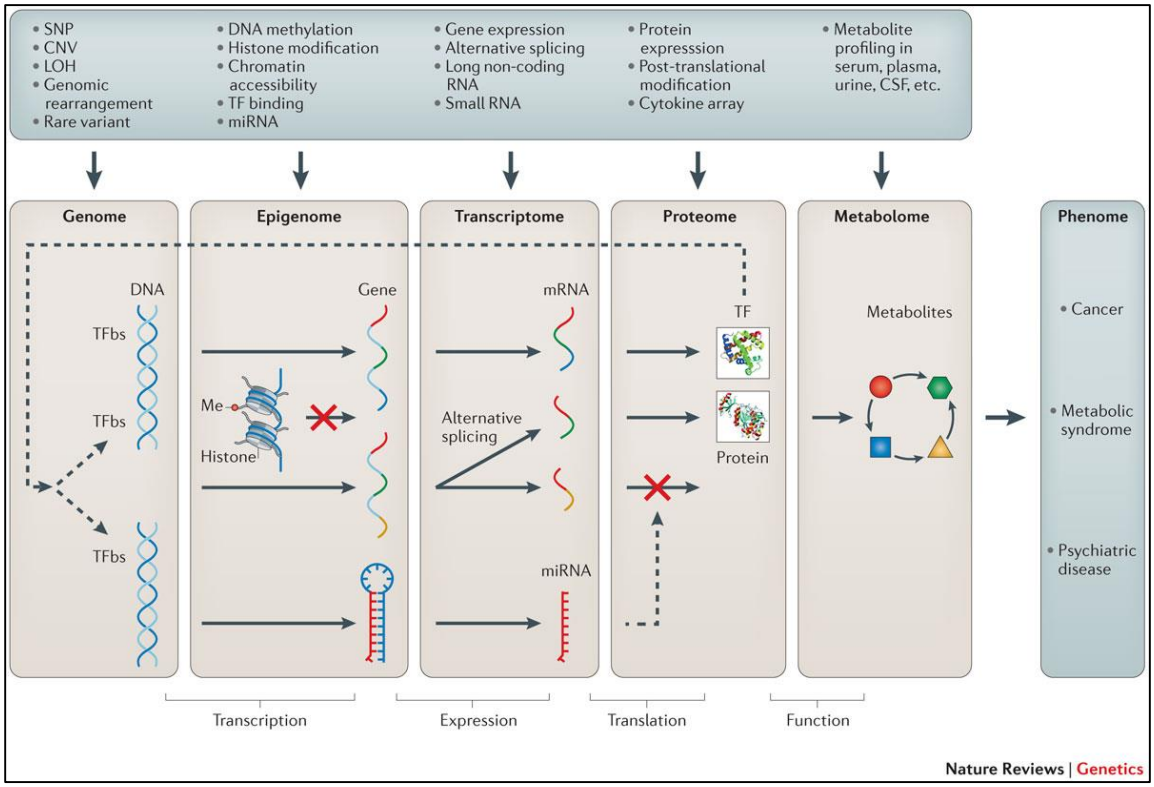

Figure 6. Heterogeneous genomic data exist within and between levels; for example, single-nucleotide polymorphism (SNP), copy number variation (CNV), loss of heterozygosity (LOH) and genomic rearrangement, such as translocation, at the genome level; DNA methylation, histone modification, chromatin accessibility, transcription factor (TF) binding and micro RNA (miRNA) at the epigenome level; gene expression and alternative splicing at the transcriptome level; protein expression and post-translational modification at the proteome level; and metabolite profiling at the metabolome level. Arrows indicate the flow of genetic information from the genome level to the metabolome level and, ultimately, to the phenome level. The red crosses indicate inactivation of transcription or translation. CSF, cerebrospinal fluid; Me, methylation; TFBS, transcription factor-binding site (Ritchie et al., 2015).

Omic technology, now referred to as 'high-dimensional biology', is commonly applied to broaden the understanding of physiological process, disease processes (i.e., screening, diagnosis, prognosis, and etiology), and drug discovery (i.e., biomarker, toxicity, and efficacy). Data analysis and integration from omics need to be managed efficiently in order to interpret the hypothesis and deliver accurate and robust results. As a 'discovery tool', it allows for a universal characterization of changes in biological variables (Karahalil, 2016; Romero et al., 2006).

1.4.1. Genomics

Genomics is the systematic study of genome – the complete genetic material in the chromosomes of an organism, containing its nucleotide sequences, genes, and genome structure and composition. Analysis of DNA sequences allows the detection of genetic variants between individuals and the identification of genes. This can be done either by Sanger sequencing or by high-throughput methods like next generation sequencing (NGS) or third generation sequencing (TGS). Although considered as conventional, Sanger method is the gold standard amongst the said strategies. This method is based on selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerase during in vitro DNA replication (Horgan & Kenny, 2011; Jamuar et al., 2016; Karahalil, 2016). Development in sequencing strategies pave the way for 'next-generation' sequencing technologies. These NGS offer cost-effective sequence throughput that are commercially available today (i.e., Illumina (Solexa) sequencing, Roche 454 sequencing, Ion torrent: Proton/PGM sequencing, and SOLiD sequencing). The resurgence of third-generation DNA sequencing and mapping technologies provides high-quality genome sequencing. In comparison with NGS, TGS can generate longer base-pair reads and maps. This single-molecule sequencing development allows high-quality detection and analysis genome structure.

The pig (*Sus scrofa*) genome has been mapped by segregation analysis of 239 genetic markers through the PiGMaP (Pig Genome Mapping Project) consortium (Archibald et al., 1995). The pig (*Sus scrofa*) has 18 autosomes in its genome, along with

X and Y sex chromosomes. With a genome size of about 2.7Gb, it is comparable to the size of human genome. The domestic pig belongs to the *Suidae* family, a group of pig species from the Cetartiodactyla order that first appeared between 20 and 30 million years ago. It is the only domesticated species in this family, which includes wild boars and domestic pigs (Frantz et al., 2016). Following the successful generation of the pig's genetic and physical maps, the Swine Genome Sequencing Consortium (SGSC) was established in September 2003 to begin sequencing the pig's genome (Schook et al., 2005). The SGSC's approach relied on hierarchical shotgun Sanger sequencing of bacterial artificial chromosome (BAC) clones, which provide a minimal tile path across the genome. Later, Illumina next-generation sequencing data was included as a supplement (Archibald et al., 2010; Humphray et al., 2007). In 2012, a draft reference genome sequence for *S. scrofa* was assembled and published as a result of these efforts. Along with this reference genome sequence, which came from a female Duroc pig, the SGSC also detailed the genome sequences of 48 other pigs from other breeds and wild boars (Groenen et al., 2012). Additionally, a *de novo* assembled genome of a Tibetan wild boar and an independent genome assembly of a Chinese Wuzhishan minipig, based on Illumina short reads, were reported in 2013 and 2014, respectively (Fang et al., 2012; M. Li et al., 2013; Vamathevan et al., 2013). Since then, the genomes of a large number of individual pigs have been re-sequenced in order to understand the genome variation, evolution, and selection in this species. At the moment, 350 entire pig genomes are readily available to the general public (Groenen, 2016). Furthermore, this has produced linkage maps that helped in the identification of quantitative trait loci (QTLs) in pigs.

Pig genetic potential can have a significant impact on a pig’s productivity and profitability. Litter size, growth rates, and carcass quality are all influenced by a combination of genetics, diet, health, environment, and management. Best Linear Unbiased Prediction, or BLUP, is a methodology that can speed up genetic advancement by providing a more precise assessment of each animal's breeding value (Lillehammer et al., 2011). Genetic improvement has been evident through years of selection and breeding strategies (Table 3). Early in the 20th century, elite breeding stock was recognized with rewards based on the physical traits and breed standards. Because of this, the breeding

objectives and phenotypes placed a greater emphasis on exterior features. Crossbreeding and specialized sire and dam lines were developed later on. Additionally, to meet the growing demand for leaner pork, breeding objectives began to place more emphasis on lowering backfat and improving growth rates or days to market. Genetic advancements in reproduction features, particularly litter size at birth, have been developed since the 1990s. More advancements have been made to include additional phenotypes, such as weaning to estrous interval, the number of teats, the color of the meat, the water binding capacity, and marbling (Merks et al., 2012). Since the early 1990s, pig breeders have been using gene marker technology to eliminate deleterious genes like the halothane gene (HAL), which causes porcine stress syndrome (PSS), and the rendement napole gene (RN^-), which causes to cause low ultimate pH and water holding capacity (WHC) in pork carcass (Hamilton et al., 2000).

Table 3. Development of pig performance from 1960s to present (van der Steen et al., 2005).

Trait	Performance		
	1960s	**Present**	**% change**
Pigs weaned/sow/year	14	21	50
Lean, %	40	55	37
Feed conversion ratio (FCR)	3.0	2.2	27
Lean meat, kg/t of feed	85	170	100

Prior to the introduction of interval mapping of QTL by Lander and Botstein at the end of the 1980s, the infinitesimal model of quantitative characteristics had dominated quantitative genetics for more than 70 years (Lander & Botstein, 1989). According to the infinitesimal model, which is also known as the polygenic model, a quantitative trait is regulated by an infinite number of *loci*, each of which has an indefinitely little effect. As a result of this model's inability to distinguish between each *locus*' effects, these *loci* must be analyzed together within the larger context of traditional quantitative genetics (Hu et al., 2012). The infinitesimal model is essentially a continuous genome model if all the minor effect genes are placed linearly in a genome. Genomics has allowed the identification of QTLs, *loci* with a major effect on the variation of a quantitative trait, their genome

localization and effect. QTL is a genomic region that is responsible for the differences in a quantitative trait. Variations in QTL can be explained through correlation analysis of genotypic data to phenotypic data (Jin et al., 2016). Molecular markers are used to locate QTLs (microsatellites and single nucleotide polymorphisms or SNPs). QTL mapping has a power to detect genomic regions that co-segregate with a known trait in a cross between breeds or lines, or in a population. Conversely, it is then limited to only allelic diversity that segregates among the parents and the amount of recombination. On the other hand, Genome-wide association studies (GWAS) evaluates the association of genotyped marker along all the chromosomes, using a high-density SNP genotyping chip, and a desired phenotype. The completion of the pig genome sequencing and availability of the *Porcine Illumina SNP60 BeadChip* with 62,163 SNPs allowed the first GWAS in pigs. Later other genotyping chips were developed, like the *Axiom Porcine Genotyping Array* (Affymetrix) with 658,692 markers or the *GGP Porcine HD Array* with 70,000 SNPs. Peaks of significant SNPs in the GWAS are genomic regions associated with the trait, that are needed to be fine-mapped to identify the gene and causal mutation affecting the phenotype. However, only for a few number QTLs or GWAS genomic regions the gene and causal mutation have been identified.

Candidate genes (Table 4) from QTL mapping studies are identified based on information of gene function and expression and also their position within the genome. According to Pig QTLdb Release 37, there have been 28,750 pig QTLs released for public access that were derived from 646 publications and characterize 677 different pig traits. According to the database, 5,723 QTLs are associated with fat composition, 3,035 QTLs are related to fatness, 1,739 QTLs and 1,034 QTLs to growth and conformation traits, respectively (Fig. 7). It was also reported that the advantage of using genomics is that it can identify the deregulated/regulated genes and pathways, whereas, its limitation arises on the prediction of the final biological effect of the variations detected (i.e., post-transcriptional- or post-translational- changes, epigenetics) (Karahalil, 2016).

Table 4. Examples of candidate genes with reported association for meat quality and fatty acid composition (Ernst & Steibel, 2013; Muñoz et al., 2018).

Gene name	**Gene Symbol**	**Associated Trait(s)**	**Reference(s)**
Acetyl-CoA carboxylase alpha	*ACACA*	Fatness, meat quality, and FA profile	Muñoz et al., 2013
Acyl-CoA synthetase long chain family member 4	*ACLS4*	Meat quality, FA profile	Corominas et al., 2012
Adiponectin, C1Q and collagen domain containing	*ADIPOQ*	Fatness	Zhang et al., 2014
Cytochrome P450 family 2 subfamily E member 1	*CYP2E1*	Meat quality	Skinner et al., 2005; Morlein et al., 2012
ELOVL fatty acid elongase 6	*ELOVL6*	Meat quality, FA profile	Corominas et al., 2013
Fatty acid binding protein 4	*FABP4*	Meat quality, FA profile	Ojeda et al., 2006
Fatty acid binding protein 5	*FABP5*	Meat quality, FA profile	Estelle et al., 2006
Fatty acid synthase	*FASN*	Fatness, meat quality, and FA profile	Muñoz et al., 2007
Insulin-like growth factor 2	*IGF2*	Fatness, carcass composition	Van Laere et al., 2003
Leptin	*LEP*	Fatness, carcass composition	Kennes et al., 2001
Leptin receptor	*LEPR*	Growth, carcass composition	Ovilo et al., 2005; Ovilo et al., 2010
Melanocortin 4 receptor	*MC4R*	Growth, carcass composition	Kim et al., 2000
Myostatin	*MSTN*	Carcass composition	Tu et al., 2014
Microsomal triglyceride transfer protein	*MTTP*	Meat quality, FA profile	Estelle et al., 2009
Myopalladin	*MYPN*	Carcass composition	Wimmers et al., 2007
Phosphoenolpyruvate carboxykinase 1	*PCK1*	Meat quality	Latorre et al., 2016
Phosphorylase kinase catalytic subunit gamma 1	*PHKG1*	Fatness, meat quality	Ma et al., 2014
POU class 1 homebox 1	*POU1F1*	Growth, carcass composition	Kuryl and Pierzchala, 2001; Song et al., 2007

PPARG coactivator 1 alpha	*PPARGC1A*	Meat quality	Gandolfi et al., 2011
Protein kinase AMP-activated non-catalytic subunit gamma 3	*PRKAG3*	Meat quality	Milan et al., 2000; Ciobanu et al., 2001
Ryanodine receptor 1	*RYR1*	Meat quality	Fujii et al., 1991; Roberts et al., 2001
Stearoyl-CoA desaturase	*SCD*	Meat quality, FA profile	Estany et al., 2014
Titin	*TTN*	Meat quality	Wimmers et al., 2007

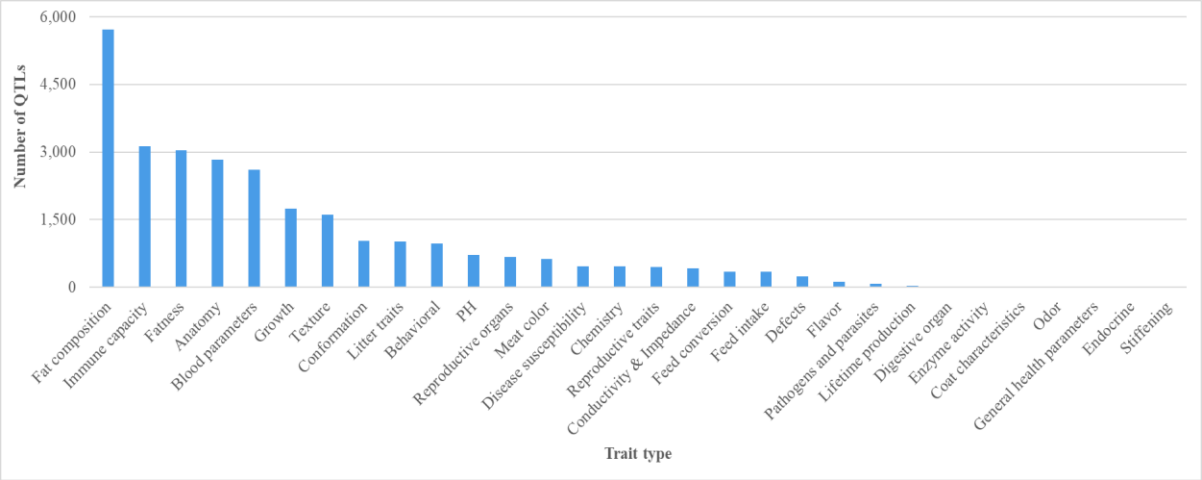

Figure 7. QTL/associations by Trait Types published in the Pig QTLdb (Hu et al., 2019).

1.4.2. Transcriptomics

Transcriptomics is the study of the transcriptome, or the entire collection of RNA transcripts produced by the genome under certain conditions or in a particular cell, utilizing high-throughput techniques like microarray analysis. The discovery of genes with differential expression in various cell groups or in response to various treatments is attained by the comparison of transcriptomes. This study is also used in predicting changes in protein levels and activities. Transcriptomics aim to catalogue all species of transcripts (i.e., mRNA, antisense RNA, and small RNA), to assess transcriptional structures and patterns, and to quantify the changing expression levels under different conditions

(Jazayeri et al., 2014; Wang et al., 2009). Linkage association between genotype and phenotype can be elucidated since mRNAs match with specific genes in the genome.

The first RNA molecule sequencing is reported in 1964. From there, new progress and development of RNA and transcriptome analysis is observed – the use of Northern Blot technique, the first reports of RT-PCR for transcriptome analysis, the introduction of Expressed Sequence Taqs (EST) sequencing, the Serial Analysis of Gene Expression (SAGE), DNA hybridization microarray, and lastly, in 2008, the first experimental report on the use of RNA-Seq (Wang et al., 2009).

DNA microarray (chips) is used to analyze a multiple number of genes in one experiment. This also takes into account changes in genes expression and DNA mutations. There are now commercially available pre-made chips that are affordable and cost-effective. Data from transcriptomics may be challenging to interpret statistically since it only provides relative abundance, and not the absolute value. (Govindarajan et al., 2012).

To aid the disadvantages of microarrays, RNA-Seq is a high throughput sequencing technology providing a survey of entire transcriptome. It can produce a more accurate result than microarrays – providing the specific site of transcription boundaries, the connection of two exons, and sequence variations in transcribed regions (Wang et al., 2009). It can also report information on alternative splicing and non-coding RNA (i.e., microRNA). One of the major challenges in RNA-Seq is the library preparation which involves the conversion of RNA to a cDNA library that will be sequenced and mapped to a reference genome.

Transcriptomics has now been used in several studies in pigs, generating valuable data for the studies on molecular mechanisms determining meat quality traits. A recent research reported on how fatty acid composition affects the porcine muscle transcriptome, detected putative proteins, and differentially expressed genes that were associated to lipogenesis and lipolysis (Puig-Oliveras et al., 2014). Another study on pig skeletal muscle observed high expression of genes such as liver X receptor α (*LXRα*) and low expression of fatty acid binding protein 3 (*FABP3*), lipoprotein lipase (*LPL*), and long-chain acyl-CoA synthetase (*ACSL*), and carnitine palmitoyltransferase 1 (*CPT1*). Such observations

indicated that there is an increased fat deposition in the muscle which is in accordance to the effects of adenosine 5'-monophosphate-activated protein kinase (*AMPK*) (Liu et al., 2018). A study on fatty acid and transcriptome profile of *longissimus dorsi* muscles between pigs breeds also showed and identified differently expressed genes (DEG) between Landrace and Lantang pigs, 13 of which is associated with fatty acid composition (i.e., *FASN*, *SCD*, ELOVL fatty elongase 2, *ELOVL2*). The effect of stearoyl-CoA desaturase (*SCD*) on fatty acid composition in muscle is highlighted due to its regulation on other desaturase or elongase required by PUFA synthesis. Likewise, together with ELOVL fatty acid elongase 6 (ELOVL6), *SCD* has a strong effect on fatty acid composition and can be used as a marker to select for optimum fatty acid profiles of pork (Corominas et al., 2015; Maharani et al., 2013; Ros-Freixedes et al., 2016). One disadvantage of transcriptomics is that it fails to account post-translational modifications which can influence the protein expression (Yu et al., 2013).

1.4.2.1. MicroRNAs and their biogenesis and mechanism of actions

MicroRNAs or miRNAs are small non-coding RNAs, usually of ~22 nucleotides long, that play key role in biological processes via gene expression regulation or mRNA translation repression (Bartel, 2004; Cai et al., 2009; Gebert & MacRae, 2019; O'Brien et al., 2018).

As shown in Fig. 8, miRNA biogenesis starts with the processing of RNA polymerase II/III transcripts post- or co-transcriptionally. The canonical pathway is the most dominant biogenesis pathway among miRNAs. The RNA polymerase II or II initiate the biogenesis of miRNA, which then generates and transcribes a long primary miRNA (pri-miRNA) that contains a 5'-cap structure and a 3'-poly(A) tail. The complementary region of the pri-miRNA forms a hairpin loop. The generated loop is recognized by the miRNA microprocessor complex that is composed of ribonuclease III enzyme Drosha and RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8), and then cleaves one strand of the stem of the pri-miRNA hairpin with a 2-bp offset, which liberates a ~60 to 70-nt stem-loop called a precursor miRNA or pre-miRNA. Once pre-miRNAs are

generated, they are exported to the cytoplasm by an exportin 5 (XPO5)/RanGTP complex. Once in the cytoplasm, the pre-miRNAs are further processed by an RNAse III-type enzyme, Dicer, to generate 22-nt miRNA duplexes. The two strands of the duplex correspond to the mature miRNA and its miRNA*, a partially complementary small RNA derived from the opposite arm of the pre-miRNA stem (Bartel, 2004; Fukunaga, 2016; Wei & Wong, 2013).

The miRNA-miRNA* duplexes are loaded into Argonaute proteins through the help of chaperone machinery HSC70-HSP90. Once the miRNA* strand is discarded, the Argonaute complex, together with the mature miRNA, develops into a functional miRNA-induced silencing complex (miRISC). The miRNAs then mediate their effects on gene expression by base-pairing with complementary sequences in the 3' UTR of the target mRNAs, resulting in silencing of the target mRNAs via Argonaut-dependent suppression of translation or degradation of the mRNAs. The directionality of the miRNA strand determines the name of the mature miRNA form. The 3p strand originates from the 3′ end of the pre-miRNA hairpin while the 5p strand arises from the 5′ end. Some studies have also detected miRNA binding sites in other mRNA regions like 5' UTR, coding sequences, and promoter regions. The binding of miRNAs to 5′ UTR and coding regions have silencing effects on gene expression while miRNA interaction with promoter region has been reported to induce transcription (Fukunaga, 2016; Wei & Wong, 2013).

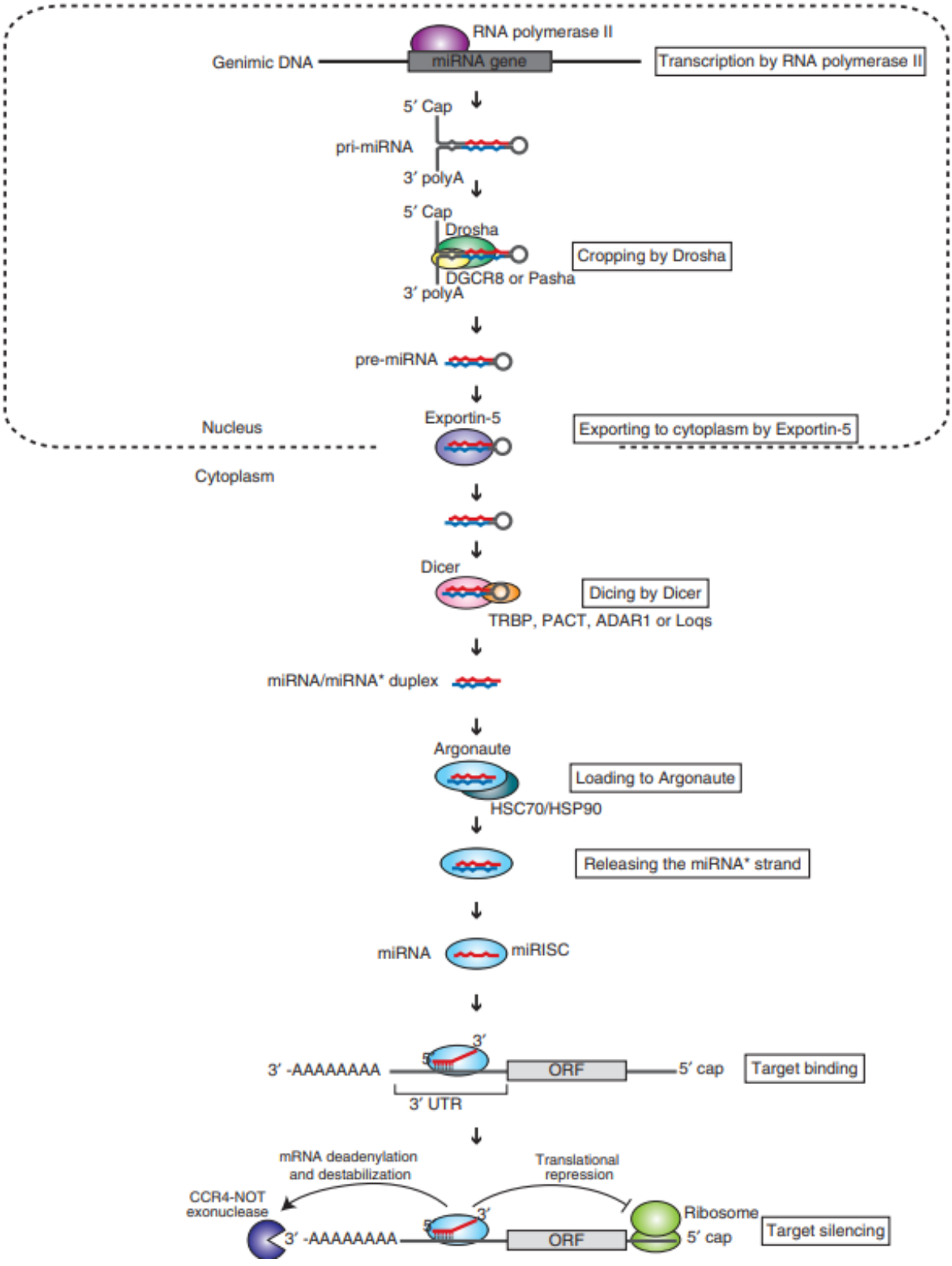

Figure 8. miRNA biogenesis and mechanism of action pathways (Fukunaga, 2016).

1.4.2.2. Exon-intron split analysis (EISA)

Recent advancements in RNA-Seq research provide reads originating from both pre-mRNA and mature RNA transcripts. Exon-intron split analysis (EISA), a novel computational method, quantifies transcriptional and post-transcriptional regulation of gene expression by comparing changes in mature RNA and pre-mRNA reads across different experimental conditions. According to this technique, it implies that the intronic counts were mainly derived from unprocessed mRNAs or pre-mRNAs and consequently indicate transcriptional modifications, whereas the difference between exonic and intronic changes allows for the calculations of post-transcriptional changes. By contrasting the quantities of exonic and intronic reads from expressed mRNA transcripts, this distinguishes the transcriptional and post-transcriptional elements of gene regulation. This technique allows us to examine on how genes are modulated at both the intron and exon levels, from being unregulated to significantly downregulated, further identify which of these genes have undergone post-transcriptional repression, and elucidate on where the where the mRNA is regulated during its life cycle (Gaidatzis et al., 2015; Mármol-Sánchez et al., 2022).

1.4.3. Proteomics

Proteomics is the large-scale study of proteome (set of proteins). This technology can give information on the specific protein expression, rates of protein production, degradation, steady-state abundance, movement, involvement in metabolic pathways, and protein-to-protein interaction (EMBL-EBI, 2019). Proteins are also exposed to post-translational modification (i.e., phosphorylation, ubiquitination, methylation, acetylation, oxidation, and nitrosylation). These modifications can have a critical effect to protein's function. Analysis of proteome can be done through Mass Spectrometry (MS)-based proteomic strategies (i.e., whole-protein and peptide analysis or top-down and bottom-up proteomics, respectively). The principle of determining proteome by using MS is to detect ions and determine their mass-to-charge information; an ion source converts the molecules

into gas-phase ions, then separate them based on m/z ratio, and record its number (Olsen et al., 2006).

Key proteins affecting muscle growth and lipid deposition were also identified in pigs. Based on experiment reported by Wang *et al*. (2017), they have found 288 differentially expressed proteins (DEPs) in skeletal muscle, 15 of which were related to lipid deposition (Wang et al., 2017). Another study by Zhong *et al.* (2011) determined the relationship between proteome changes of *longissimus dorsi* and IMF content in pigs fed conjugated linoleic acid (CLA). Results have showed that the proteins related to energy metabolism, fatty acid oxidation and synthesis, amino acid metabolism, defense, transport and other miscellaneous processes are significantly influenced by the addition of CLA (Zhong et al., 2011). There is also an observed positive correlation between IMF and increased abundance of carbonic anhydrase 3 (*CA3*) and aspartate aminotransferase (*AST*). *CA3* has known to catalyze the hydration of carbon dioxide to generate bicarbonate and hydrogen ions for fatty acids synthesis and pH homeostasis. *AST*, on the other hand, plays a role in long-chain fatty acid transmembrane movement and oxidation to provide energy, in which can be related to the fatty acid metabolism (Wang et al., 2006). These studies have shown that there is an on-going interest on the use of proteomics to study proteins related to the growth and development of pigs.

1.5. Omega-6/Omega-3 (n-6/n-3) polyunsaturated fatty acid and sow and piglet nutrition

Mammals, like pigs, cannot incorporate double bonds in fatty acids past carbon 9 and 10 as they lack required desaturase enzyme for their de novo synthesis. Hence, essential fatty acids, such as n-6 and n-3, should be supplemented in the diet (Webster, 2012). As precursors of eicosanoids, n-3 and n-6 and their antagonistic inflammatory functions and fatty acid pathways may result a substrate competition and can potentially affect metabolic health, inflammatory modulation, and homeostasis (Sakayori et al., 2020; Zivkovic et al., 2011). Eicosanoids are bioactive lipid mediators synthesized through PUFA oxygenation, majority of which are derived from n-6 PUFA. Eicosanoid signaling

has been associated as a primary pro-inflammatory component of innate immunity and can control immune system activity (Dennis & Norris, 2015; Lone & Taskén, 2013).

Commercial swine feed is mostly cereal- and soybean meal-based, accompanied with plant and plant oil sources such as sunflower oil, corn, safflower oils or other feedstuffs that are highly constituted of omega-6 (n-6) polyunsaturated fatty acids (PUFA). Consequently, this makes the amount of n-6 in feed mixes about 10 times higher than omega-3 (n-3) PUFA (Gjerlaug-Enger et al., 2015). Low dietary ratio between n-6 and n-3 (i.e., 4:1) can improve weaning weight survival, weight gain, and influence the total n-3 polyunsaturated fatty acids found in colostrum and milk (Nguyen et al., 2020). Previous human and pig studies on n-3 supplementation have shown to reduce the risk of cardiovascular diseases, obesity, and metabolic syndrome and diseases, even the placental metabolism, inflammatory status, and lipid transfer (Duan et al., 2014; Jump, 2011; Leghi & Muhlhausler, 2016; Szostak et al., 2016; Wada et al., 2017). Different sow studies found n-3 PUFA to have no effect on piglet birth weight, while positive effects on pre- and post-weaning growth have been published. This was also supported in human studies where an increased supply of n-3 was suggested to have adverse growth effects on neonates, whereas, high arachidonic acid concentration from the maternal diet have shown a positive correlation to neonatal growth (Carlson et al., 1993; Lapillonne et al., 2003). Although, n-3 in gestation diets exhibited a tendency to increase piglet birth weight and further substantiated by a meta-analysis in human studies, where n-3 PUFA addition alone improved infant birth weight and correlated to the increased concentration of n-3 PUFAs such as docosahexaenoic acid and eicosapentaenoic acid (Li et al., 2018; Rooke et al., 2001).

Sow diet influences the accumulation of fatty acids by piglets through placenta lipid transfer. Diets during gestation directly affect sow's milk and piglet's plasma fatty acid composition. This also indicates on how maternal adipose tissue acts as first depots of dietary fatty acids, which then mobilized around farrowing, and transferred to piglets (Amusquivar et al., 2010). Moreover, evidences have shown on how maternal dietary composition could possibly influence the availability and similarity of fatty acids in human placenta. Placental expression of G protein-coupled receptor 120 (GPR120, a

docosahexaenoic acid receptor) has been correlated with adipocyte differentiation in neonatal fat (Díaz et al., 2017; Wada et al., 2017). An animal study has also shown on how the inclusion of n-3 increased the growth of the fetus and placenta and reduced the oxidative degradation of lipids by increasing the expression of antioxidant enzymes in placental zones (Jones et al., 2013).

1.6. The MANNA consortium (www.phd4manna.eu)

The European Joint Doctorate in Molecular Animal Nutrition (MANNA) is an EU network whose mission is to provide a Double Doctorate level training programme, valid throughout Europe, on innovative technologies applied to animal science and nutrition. MANNA aims to provide future research leaders with the capability to address the needs to improve livestock health, welfare and production efficiency. The MANNA Joint Doctoral project is a Marie-Skłodowska Curie Innovative Training Network funded by the European Commission under the Horizon 2020 research and innovation programme H2020-MSCA- ITN- 2017- EJD: Marie Skłodowska- Curie Innovative Training Networks (European Joint Doctorate) – Grant agreement nº: 765423 – MANNA (Fig. 9).

Figure 9. The European Joint Doctorate in Molecular Animal Nutrition logo.

The structure of the programme is designed to generate an interactive network: Eleven (11) Early Stage Researcher (ESR) are supervised by two Beneficiary Universities and a non-academic participant during their PhD project. All the ESRs were thus able to spend several months in each of their supervisory institutions, which permitted them to

meet several researchers with expertise in various areas of research. This rich secondment programme permits the ESRs to further specialize in multiple fields of activities ranging from animal nutrition, veterinary diagnostic, clinical research, bioinformatics and professional communication in the agro-food sector. Moreover, all the academic and non-academic partners play a fundamental role in the training of the ESRs. Multiple events have been organized to further train and reinforce the already rich graduate program of each University involved in the framework.

The objectives of the MANNA program are as follows: (1) the creation and the development of an Elite EU School to train the ESRs in the application of OMIC technologies to animal science and nutrition; (2) Evaluate and understand the influence of innovative feed additives on animal production; and (3) Deliver a double doctoral degree and establish a new generation of scientists that will take forward this cutting-edge technology to a global workplace.

OBJECTIVES

Chapter 2

AIMS OF THE PROJECT

This PhD thesis was done under the framework of the European Joint Doctorate in Molecular Animal Nutrition (MANNA), a Marie-Skłodowska Curie Innovative Training Network funded by the European Commission under the Horizon 2020 research and innovation program H2020-MSCA- ITN- 2017- EJD: Marie Skłodowska- Curie Innovative Training Networks (European Joint Doctorate) – Grant agreement nº: 765423 – MANNA. A part of this research was supported by the Spanish Ministerio de Ciencia e Innovación (MICINN) and the Fondo Europeo de Desarrollo Regional (FEDER) with project references: AGL2017-82641-R and PID2020-112677RB-C22. The present research has been performed using the animal materials generated by both the IBMAP project and MANNA program (a project involving INIA, IRTA, UAB, and UNIMI).

The main objective of the project is to determine the effect of dietary lipids, in terms of omega-6/omega-3 polyunsaturated fatty acid ratio, on porcine meat quality relating to intramuscular fat content and composition. Specifically, it aimed to:

1. To identify differentially expressed (DE) genes, microRNAs (miRNAs), and biological pathways related to changes in the n-6/n-3 PUFAs muscle composition in pigs and assess mRNA-miRNA transcriptomic interactions using computational prediction and regulatory network analyses (Paper I).
2. To determine on how the sow's milk, fed with high or low n-6/n-3 polyunsaturated fatty acid (PUFA) ratio diets, directly influences the fat deposition in muscle and in adipose tissues of pre-weaned piglets and the expression profiles of genes, abundance of proteins, and their related ontologies and biological pathways (Paper II).
3. To determine post-transcriptional (PTc) regulation on *longissimus dorsi* muscle using exon-intron split analysis (EISA) in male and female piglets from sows fed with high and low n-6/n-3 PUFA ratio diets and correlate both gene and miRNA expression profiles to the PUFA profile of skeletal muscle (Paper III).

PAPERS AND STUDIES

Chapter 3

PAPER I

Identifying miRNA-mRNA regulatory networks on extreme n-6/n-3 polyunsaturated fatty acid ratio expression profiles in porcine skeletal muscle

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Abstract

Omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFAs) are essential fatty acids with antagonistic inflammatory functions that play vital roles in metabolic health and immune response. Current commercial swine diets tend to over supplement n-6 PUFAs, which may increase the likelihood of developing inflammatory diseases and affect the overall well-being of the animals. However, it is still poorly understood how n-6/n-3 PUFA ratios affect the porcine transcriptome expression and how messenger RNAs (mRNAs) and microRNAs (miRNAs) might regulate biological processes related to PUFA metabolism. On account of this, we selected a total of 20 Iberian x Duroc pigs with extreme values for n-6/n-3 FA ratio (10 high vs 10 low), and *longissimus dorsi* muscle samples were used to identify differentially expressed mRNAs and miRNAs. The observed differentially expressed mRNAs were associated to biological pathways related to muscle growth and immunomodulation. The differentially expressed microRNAs (*ssc-miR-30a-3p, ssc-miR-30e-3p, ssc-miR-15b* and *ssc-miR-7142-3p*) were correlated to adipogenesis and immunity. Relevant miRNA-to-mRNA regulatory networks were also uncovered (i.e., *mir15b* to *ARRDC3*; *mir-7142-3p* to *METTL21C*), and linked to lipolysis, obesity, myogenesis, and protein degradation. The n-6/n-3 PUFA ratio differences in pig skeletal muscle revealed genes, miRNAs and enriched pathways involved in lipid metabolism, cell proliferation and inflammation.

Keywords: microRNA; mRNA; regulatory network; PUFA; pig

Introduction

Dietary concentration of polyunsaturated fatty acids (PUFA) can potentially affect and change the gene expression profile of key tissues such as skeletal muscle or fat compartments, with relevant implications for their commercial transformation and consumption (1). These alterations, due to nutritional interventions, may rewire multiple regulatory networks in nutrient metabolism, thus affecting messenger RNA (mRNA) transcription, splicing, trafficking and further synthesis of derived proteins (2,3). Gene expression regulation can also be mediated through post-transcriptional regulation, of which microRNAs (miRNAs) are key effectors. miRNAs are small non-coding RNAs of ~22 nucleotides long that are able to bind to specific sequences of the 3' untranslated regions (3' UTRs) of targeted mRNAs and trigger their degradation and/or inhibit their translation (4). Thousands of miRNAs can be found on online databases such as *miRBase*, a searchable collection of published miRNA sequences and their annotation. As of its release v22.1, there are 38,589 miRNA entries published over 271 species, including 408 precursors and 457 mature miRNAs for *Sus scrofa* (pig) (5). Besides, the development of next generation sequencing (NGS) technologies has provided a better understanding of the genome organization, structure, function, and evolution in livestock animals. Nowadays, it is commonly used to study complex traits to improve livestock production efficiency and reproductive health (6).

Pigs are one of the most important agricultural livestock animals for meat production, accounting to a total of 122.5 million tons globally in 2021. Food and Agriculture Organization (FAO) also highlighted that the recent expansion of world meat output was mainly driven by the increase in pork output (7). Porcine fatness or leanness are considered as relevant target traits for selection since they could impact productive and reproductive performance, as well as meat quality (8). Specific porcine breeds, such as Landrace, have been extensively selected to increase lean meat production and reduce fat deposition (9). Although this may improve overall pig production efficiency, such breeding programs may negatively affect meat quality traits like juiciness, tenderness, flavor and overall sensory quality of pork (10). For a more efficient production, fats and oils are

supplemented on diets, as they contain 2.25 times more energy than cereal grains, which will further increase energy density and reduce feed intake (11). Additionally, the fatty acid content of the carcass is directly influenced by the dietary fats ingested by pigs, mimicking the fatty acid composition of the diet (12). While most of the porcine mRNAs and miRNAs have been identified from skeletal muscle, liver, and adipose tissue, among others, there is still limited knowledge on how mRNAs and miRNAs interact to regulate fatty acid metabolism pathways, or how diet and fatty acid content might influence their expression profiles, especially on Iberian pigs (13). Moreover, as the excessive supplementation of omega-6 (n-6) PUFAs becomes more prevalent on commercial pig diets, this pro-inflammatory PUFAs can impose risk of developing inflammatory diseases such as cardiovascular diseases, diabetes or obesity (14,15). Regulation of n-6 PUFA-derived metabolites can be done through balancing the ratio between these and omega-3 (n-3) PUFAs, which counteract pro-inflammatory responses elicited by the excess of n-6 PUFAs (16).

In order to better understand putative regulatory relationships between mRNAs and miRNAs related to changes in the n-6/n-3 PUFAs muscle composition in pigs, we identified differentially expressed (DE) mRNAs and miRNAs in skeletal muscle tissue from a population of Iberian x Duroc pigs with high and low values of n-6/n-3 PUFAs ratio. Moreover, we assessed mRNA-miRNA transcriptomic interactions using computational prediction and regulatory network analyses.

Materials and methods

Animal material

A total of 20 *longissimus dorsi* (LD) skeletal muscle samples were obtained from an experimental backcross population of Iberian and Duroc pigs, as previously described by Martinez-Montes *et al*. (17). Pigs were housed following standard intensive system according to European directives on animal welfare and were fed *ad libitum* with a cereal-based commercial diet. Muscle samples were collected immediately after slaughter, snap-

frozen in liquid nitrogen and stored at -80ºC. Fatty acids profiling was performed by using gas chromatography of methyl esters protocol on 200 g of LD muscle. Sampled animals were selected based on their analyzed values for n-6/n-3 PUFAs ratio and a total of 10 with highest (H) and 10 with lowest (L) n-6/n-3 ratio values were kept for further analyses (18). A summary of the measured phenotypes in the selected animals is available at S1 Table.

Ethics Statement

All animal procedures were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in experimentation. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria CEEA (Permit Number: 2014/026).

RNA isolation, library preparation, and sequencing of total and small RNAs

Total RNA

The LD skeletal muscle samples were submerged in liquid nitrogen, pulverized using a mortar and pestle, and subsequently homogenized in 1 ml of TRI Reagent (Thermo Fisher Scientific, Barcelona, Spain). The RiboPure kit (Ambion, Austin, Texas, USA) was used to isolate the total RNA fraction, and its concentration and purity were determined with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Barcelona, Spain). RNA integrity was assessed with a Bioanalyzer-2100 equipment (Agilent Technologies Inc., Santa Clara, California, USA), using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Inc., Santa Clara, California, USA). Libraries were prepared with the TruSeq SBS Kit v3-HS (Illumina Inc., California, USA) and a minimum of 30 million hits of 75 bp-length paired-end reads were acquired per sample using an Illumina HiSeq

3000/4000 equipment (CNAG-CRG, Barcelona, Centro Nacional de Análisis Genómico; https://www.cnag.crg.eu).

Small RNA

The extraction of total RNA, including miRNA and small RNA, was performed using the same muscle tissue material and employing the miRNeasy Kit (QIAGEN, Germantown, Maryland, USA) following manufacturer's specifications. Approximately 50 mg of tissue samples were disrupted and homogenized in 700 µl of QIAzol Lysis Reagent (QIAGEN, Germantown, Maryland, USA) using 2ml Lysing matrix D tubes (MP Biomedicals, Santa Ana, CA) and a Precellys 24 instrument (Bertin Technologies, Rockville, MD). After following the miRNeasy protocol, RNA was eluted with 30 µl of water. The concentration, purity, and RNA integrity were assessed as per aforementioned for total RNAs. A minimum of 10 million hits of 50 bp-length single-end read were acquired per animal using the same sequencing equipment used for mRNA libraries.

RNA-Seq and miRNA-Seq data processing

Raw mRNA and miRNA sequences were subjected to quality control through the FastQC tool (19). In order to remove the Illumina adapters used during library preparation and sequencing, reads were trimmed using the Cutadapt software v0.9.5 (20). RNA-Seq data sequence alignment was performed against the reference pig genome (*Sscrofa11.1*) by using the STAR (21) aligner. Sequences were then quantified through RSEM software (22). On the other hand, for miRNA-Seq data, sequence alignment was performed against the reference pig genome (*Sscrofa11.1* and miRBase 22.1) by using Bowtie[23] aligner and the following specifications for aligning short miRNA reads were taken into consideration: 1) allowing no mismatches in the alignment, 2) removing reads with more than 20 putative mapping sites and 3) reporting first single best stratum alignment (bowtie -n 0 -l 25 -m 20 -k 1 --best –strata) (23,24). Quantification of aligned miRNA reads were performed using

HTSEQ (25). Only mRNA and miRNA with an overall expression across samples higher than 20 counts were considered for differential expression analyses (26).

Differential gene expression analyses between the H and L groups of animals from both RNA-Seq and miRNA-Seq data were performed with the DESeq2 software (26) and correcting for sex and batch covariates (S1 Table). Both mRNAs and miRNAs from differential expression analyses were considered significant at a fold change > |1.5| and adjusted *p*-value < 0.05. We considered the H group as base control, meaning that any gene upregulation would imply its overexpression in L group, resulting in a positive fold change and vice versa.

Gene ontology and pathway enrichment analysis

Differentially expressed mRNA genes analyzed between H and L groups were subjected to Gene ontology (GO) and pathway enrichment analyses using Cytoscape v3.7.1 software with the ClueGO v.2.5.4 plug-in application to determine enriched Biological Process terms (27,28). Identification of enriched terms was done using a two-sided hypergeometric test of significance, with a false discovery rate approach for multiple testing correction (29).

Co-expression network analysis between mRNA and miRNAs

A co-expression network between mRNA and miRNA expression profiles was built according to the established pipeline as previously reported by Mármol-Sánchez *et al.*(24). The Partial Correlation with Information Theory (PCIT) network inference algorithm was used to recognize meaningful gene-to-gene interactions by employing first-order partial correlation coefficients obtained for each trio of genes in conjunction with an information theory technique (30,31). To do so, we calculated the Pearson pairwise correlation coefficients (*r*) for each expressed miRNA and DE mRNA between H and L groups. Assuming that miRNAs can biologically suppress mRNA expression, we reported only

those co-expressed pairs of miRNA and mRNAs showing an *r* value < -0.5. To further retain only relevant miRNA-to-mRNA correlations with biological meaning, the seed portions of the annotated porcine mature miRNAs (7mer-m8, from 2nd to 8th 5' nucleotides) were reverse-complemented and interrogated along the annotated 3' UTRs (Sscrofa 11.1; http://www.ensembl.org./biomart) of porcine mRNA genes, by making use of the SeqKit toolkit (32). We also looked at whether the mRNAs predicted to interact with miRNAs showed meaningful expression correlations with other mRNA-encoding genes. We only kept the mRNA pairs with $r > |0.7|$ as determined by the PCIT algorithm, because the correlation expression between mRNA pairs is commonly of great magnitude than that of miRNA-mRNA interactions (33).

Results

Differentially Expressed Genes (DEGs) and miRNAs (DEmiRNAs)

Out of 11,521 porcine mRNAs detected as sufficiently expressed, a total of 432 DEGs were obtained between H and L pigs according to their n-6/n-3 PUFA ratio (**S2 Table**), with 157 and 275 genes being upregulated and downregulated in L pigs with respect to H pigs, respectively (Fig 1). From the 457 annotated porcine miRNAs, a FC > |1.5| threshold for changes in expression between H and L pigs showed no DE miRNAs for the PUFA ratio trait. When a less stringent FC threshold (F| > |1.2|) was considered, 4 DEmiRNAs were recovered: *ssc-miR-15b, ssc-mir30a-3p, ssc-miR-30e-3p* and *ssc-miR-7142-3p* (S3 Table).

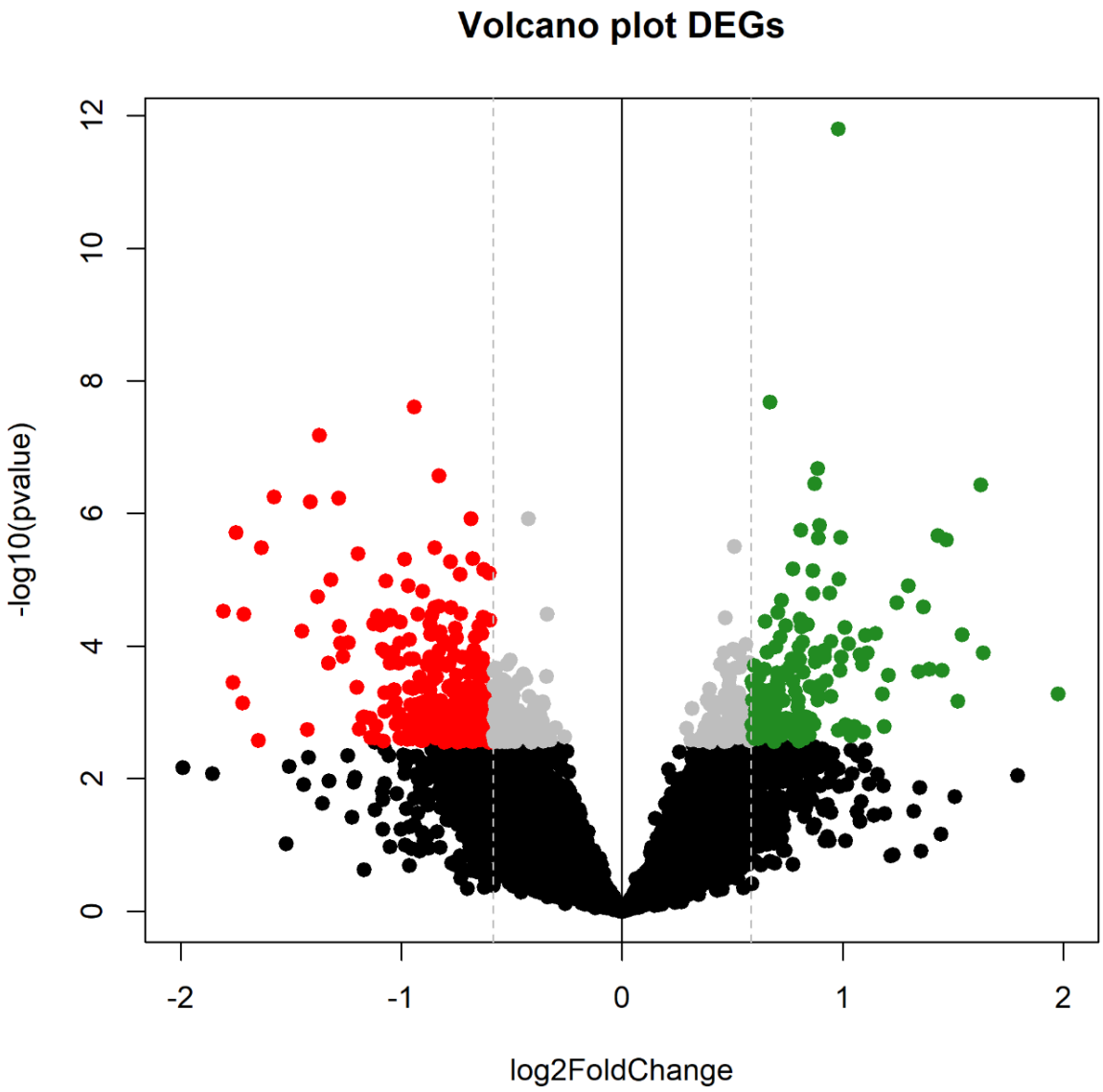

Figure 1. Volcano plot showing differentially expressed genes (DEGs) with fold change > |1.5| and *p*-adj value < 0.05) after comparing pigs with High (H) and Low (L) profiles of n-6/n-3 PUFAs ratio in *longissimus dorsi* skeletal muscle. Upregulated genes (green) correspond to genes overexpressed in L pigs and vice versa.

Functional analysis and pathway enrichment of DEGs

A total of 80 significant unique GO terms (*p*-adj value < 0.05) were detected for DEGs related to H and L pigs for the n-6/n-3 PUFA ratio trait. A full list of enriched GO

terms is shown in S4 Table. These gene ontologies and biological pathways were related to muscle structure development (*GO:0061061*), positive regulation of skeletal muscle cell differentiation (*GO:2001016*), SREBP signaling pathway (*GO:0032933*) and adrenergic receptor signaling pathway (*GO:0071875*), as shown in Fig 2.

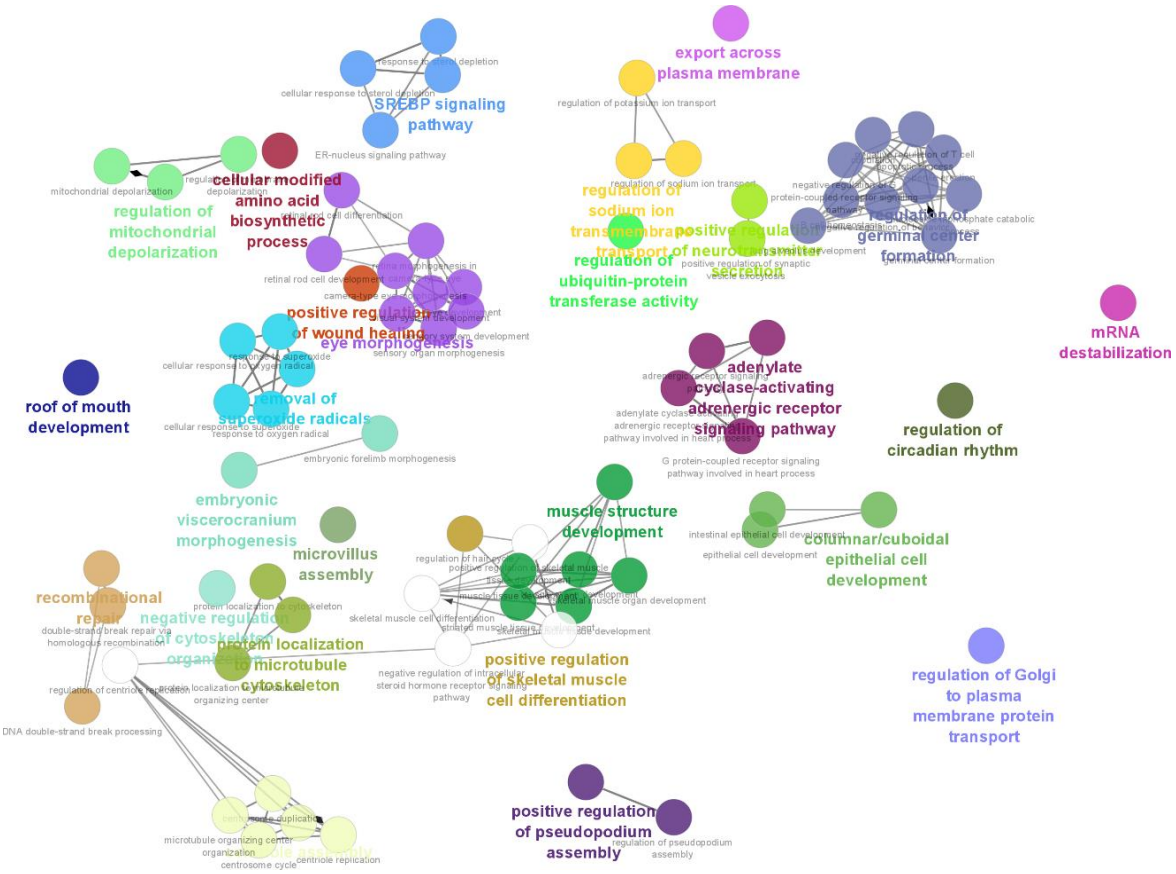

Figure 2. Enriched Gene Ontologies using DEGs after comparing RNA-Seq gene expression profiles of H and L pigs and their related biological processes. Significant unique GO terms (p_{adj}-value < 0.05) obtained from the ClueGO plug-in embedded on Cytoscape software.

mRNA-miRNA co-expression regulatory network

A total of 196 miRNAs were detected as co-expressed (r < -0.50) with the differentially expressed mRNA genes for n-6/n-3 PUFAs ratio (S5 Table). Among the 196

detected miRNAs, we focused on the 4 DEmiRNAs for the same trait (S3 Table) and used them for interrogating miRNA seed binding sites to the 3' UTR region of putative DE mRNA targets (S6, S7, and S8 Tables). Almost half (214 out of 432) of the DEGs showed putative binding sites in their 3' UTRs for the seed region of the DEmiRNAs (S3 and S1 Tables).

Table 1. Number of putative targeted DE mRNAs (DEGS) with predicted binding sites for DEmiRNAs between pigs with High (H) and Low (L) n-6/n-3 PUFAs ratio in *longissimus dorsi* skeletal muscle.

DEmiRNAs	Number of targeted DEGs[a]	% over total DEGs
ssc-mir-15b	125	28.94%
ssc-miR-30a-3p	130	30.09%
ssc-miR-30e-3p		
ssc-miR-7142-3p	54	12.50%

[a] Differentially expressed genes (DEGs) = 432 in total; The *ssc-miR-30a-3p* and *ssc-miR-30e-3p* have the same mature miRNA seed (7mer-m8, from 2nd to 8th 5' nucleotides).

Further combining relevant miRNA-mRNA expression correlations according to the PCIT algorithm ($r < -0.50$) and 3' UTR region seed matching, 2 out of the 4 DEmiRNAs showed meaningful co-expression with two DEGs: *ssc-miR-15b* was predicted to bind to the 3' UTR of the arresting domain containing 3 (*ARRDC3*) gene, while *ssc-miR-7142-3p* was predicted to bind the 3' UTR of the methyltransferase-like 21C (*METTL21C*) gene (S9 Tables).

Several other genes were also significantly associated with the expression of these two DEGs (*ARRDC3* and *METL21C*, S10 and S11 Tables). As shown in Fig 3, the *ARRDC3* gene showed meaningful correlation with 41 differentially expressed mRNAs (S10 Table), whereas the *METTL21C* gene was significantly correlated with 5 DEGs (S11 Table). The related functions and associations to lipid metabolism, immunity, and/or inflammation of these DEGs were summarized in S12 Table.

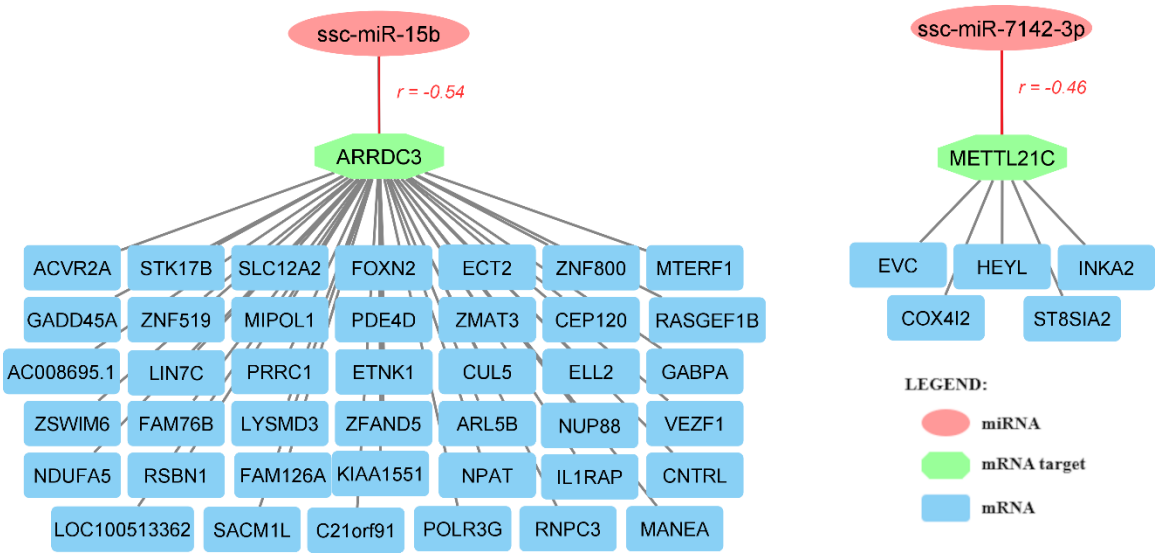

Figure 3. Meaningful co-expression network between miRNA-to-mRNA and mRNA-to-mRNA genes. *r* = Pearson correlation value; lines in red for miRNA-to-mRNA interactions denote a negative correlation.

Discussion

DEGs and their relationship to lipid-mediated expression and immunomodulation

Our results after GO enrichment analyses of DEGs (S4 Table) showed GO terms mostly related to muscle growth and differentiation, glucose and lipid metabolism. Some of the genes related to muscle tissue and structure development pathways were also reported in human, mice and ruminants (i.e., cattle, sheep, lamb). The aryl hydrocarbon receptor nuclear translocator like (*ARNTL*), a gene that regulates the circadian release of PUFAs and modulates feeding behavior in mice, alongside with forkhead box N2 (*FOXN2*), are associated with obesity (34,35). Another interesting gene was the diaphanous related formin 1 (*DIAPH1*), which is regulated upon nutritional intervention with long chain PUFAs (n-6 and n-3) and it is reported to be involved in lipid metabolism in cattle (36). Supplementation of flaxseed or fish oil can increase the expression of the guanidinoacetate N-methyltransferase (*GAMT*) gene, which is involved in folate-

homocysteine metabolism in embryos and liver of pregnant mice (37). The absence of n-3 PUFA in rodents (i.e. DHA) has been reported to affect cognitive brain function and a few of its synaptomes including homer scaffold protein 1 (*HOMER1*) (38). The Kruppel like factor 5 (*KLF5*) gene regulates muscle differentiation in myoblasts and controls lipid metabolism in mature skeletal muscle in mice (39,40). In addition, GWAS analyses in human metabolic syndrome discovered the association of the strawberry notch homolog 1 (*SBNO1*) gene on plasma high-density lipoprotein cholesterol concentration, whereas the vestigial like family member 2 (*VGLL2*) gene was linked to the fatty acids profile in sheep (41,42). The T-box transcription factor 1 (*TBX1*) gene, together with *miR-193a-3p*/*TGF-β2*, was found to drive iron-dependent cell death ferroptosis through the accumulation of lipid peroxides in neonates (43,44). Abundancy on n-3 PUFA in cattle is reported to increase the gene expression of insulin-like growth factors such as the insulin-like growth factor binding protein 5 (*IGFBP-5*) and further influence reproductive performance (45). Other genes like the glycerophosphocholine phosphodiesterase 1 (*GPCPD1*), 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*), or phosphoglucomutase 5 (*PGM5*), are related to glycophospholipid formation, cholesterol synthesis and glycolysis (46–49).

Another relevant GO term that can be highlighted from our results is the SREBP signaling pathway. Sterol regulatory-element binding proteins (SREBPs) are transcription factors that regulate the expression profiles of genes that are involved in lipid synthesis, energy storage and cholesterol regulation. When these proteins are activated, they can trigger lipid-mediated cellular stress that can cause metabolic diseases such as obesity, atherosclerosis, diabetes mellitus, inflammation, and organ fibrosis (50,51).

On the other hand, our enrichment analyses also emphasized the adenylate cyclase-activating adrenergic receptor signaling pathway based on the DEGs involved. Adrenergic receptors play a vital role in mediating stress-induced signals, in immunomodulation and in stress-related behavioral changes (52,53). This pathway also triggers the formation of cyclic-adenosine 3′,5′-monophosphate (cAMP), which regulates intracellular metabolism and it is linked to glycolysis (54,55). Stimulation of both SREBP signaling pathway and adrenergic receptor signaling pathway could also be related to the pro-inflammatory role of n-6 PUFA. Addition of n-6 PUFA increased the β-adrenergic receptor binding and

adenylate cyclase activity in pig adipocyte plasma membrane (56). Furthermore, it was also reported that over supplementation of n-6 PUFAs in swine diets can stimulate the innate immune response and acute inflammatory response (57).

Association of differentially expressed porcine miRNAs to adipogenesis and inflammation

We obtained a total of 4 DEmiRNAs (*ssc-miR-30a-3p, ssc-miR-30e-3p, ssc-miR-15b* and *ssc-miR-7142-3p*) between high and low n-6/n-3 PUFA ratio contrast on porcine skeletal muscle. The expression of *miR-30a* in pigs has been associated to adipocyte formation, fat deposition, myogenic differentiation and immune system (58–62). *miR-30a* may also be related to cellular response to infection, immune modulation and pathological processes since it was detected on multiple pig-related viral studies concerning porcine parvovirus, porcine reproductive and respiratory syndrome virus or H1N1 swine influenza A virus (63–65). A study on a minipig obesity model also demonstrated how *miR-30a* could regulate the expression of genes related to adipogenesis and low-grade chronic inflammation in obesity (58,66,67). A similar result was previously reported by our team, in which we predicted that *miR-30a* could potentially bind to and regulate the mRNA of porcine *ELOVL* fatty acid elongase 6 (*ELOVL6*) gene, which is responsible of the elongation of PUFAs and *de novo* lipogenesis (68). As a member of *miR-30* family, *ssc-miR-30e* also targets mRNA genes that are related to skeletal muscle growth, energy metabolism and increased feed efficiency in swine (69,70) A few reports on pigs have also elucidated the role of *miR-30e* on binding to mRNA transcripts from genes related to pathogenesis, virus-host interactions and immune response (71–73).

On the other hand, *mir-15b* is mainly associated to blood vessel formation (angiogenesis), tumor growth and cellular ATP level modulation. Metabolites obtained from n-6 PUFAs could promote angiogenesis by increasing expression of transcription growth factors (i.e. *TGF-β*), whereas n-3-PUFA-derived substances contain anti-angiogenic, anti-inflammatory and antitumor properties (74–76). Besides, *ssc-mir-7142-*

3p is a mirtron located in the intronic fraction of the microtubule affinity regulating kinase 2 (*MARK2*) gene. This miRNA has been detected in lung tissue infected with *Actinobacillus pleuropneumoniae* and its differential expression has been associated to the overexpression of the retinol binding protein 4 (*RBP4*) gene (77,78). *RBP4*, mainly secreted by the liver and adipocytes, is a transporter of vitamin A and it is involved in various pathophysiological processes, such as obesity, insulin resistance and cardiovascular diseases (78).

Meaningful miRNA-to-mRNA regulatory networks affected by changes in n-6/n-3 ratio

Co-expression network analyses between DEmiRNAs and DEGs highlighted 2 miRNAs that can potentially bind to and inhibit the expression of 2 DE mRNAs when comparing pigs with high and low n-6/n-3 PUFA ratio in skeletal muscle. The upregulated DEmiRNA *ssc-miR-15b* was predicted to bind to the 3' UTR of the arrestin domain containing 3 (*ARRDC3*) gene. Arrestins are a small family of multi-faceted protein trafficking adaptors that bind to membrane proteins, which regulate signal transduction at G protein-coupled receptors (GCPR) and promote endocytosis. *ARRDC3* is a known α-arrestin and its activation could be due to nutrient excess or cellular stressors (79,80). Our results showed that this gene was involved in a few gene ontology pathways such as adrenergic receptor signaling pathway, negative regulation of G protein-coupled receptor signaling pathway, negative regulation of behavior and regulation of ubiquitin-protein transferase activity. *ARRDC3* was reported to co-immunoprecipitate and interact with β_2-adrenergic receptors and facilitate its ubiquitination and degradation (81–83). In addition, this gene is also involved in obesity development, insulin resistance, body mass regulation, glucose metabolism, adiposity and energy expenditure (84–86).

Meanwhile, the mirtron *ssc-miR-7142-3p* might target the methyltransferase-like 21c (*METTL21C)* mRNA transcripts, which encode for a protein-lysine methyltransferase involved in regulation of myogenesis, muscle function and protein catabolism (87,88).

From our results, we found that high n-3 PUFA concentration tends to upregulate the expression of *METTL21C*. A decreased expression of this gene was also reported after long-term exercise, in which elevated levels of inflammatory cytokines, oxidative stress, and leukocytosis could be observed (89,90). From our results, we might hypothesize that the upregulation of *ssc-miR-15b* and downregulation of *ssc-miR-1472-3p*, together with *ARRDC3* downregulation and *METTL21C* upregulation, in presence of low n-6/n-3 PUFA ratio, could be linked to pro-inflammatory metabolites stimulating receptors related to stress and immunity. However, further validation among these predicted regulatory networks should be done in order to verify their biological importance in terms of porcine growth and immune response.

Putative mRNA-to-mRNA correlations highlight genes related to lipid-mediated biological processes and immunity

Potential correlation and interaction between the two possible target genes of DEmiRNAs, *ARRDC3* and *METTL21C*, and DEGs were further investigated. The phosphodiesterase 4D (*PDE4D*), a gene that is associated with the regulation of interleukin production and cAMP-mediated signaling, belongs to the same adrenergic receptor signaling pathway as *ARRDC3* (S4 Table). The tumor inhibition properties of *ARRDC3* are presumably facilitated by linking target substrates such as β-adrenergic receptor and integrin β4 to E3 ligase, in which these target substrates become ubiquintinated and degraded by the proteasome (91).

Both the Hes related family bHLH transcription factor with YRPW motif like (*HEYL*) and EvC ciliary complex subunit 1 (*EVC*) genes were correlated with *METTL21C* and associated with muscle organ and structure development (S4 Table). One study looked into the changes of gene expression on some signaling pathways that could be affected by the specific knockdown of *METTL21C*, including *HEYL*-targeted Notch pathway. Although it did not affect the expression of *HEYL* gene, they reported that *METTL21C* is a critical component for bone and muscle homeostasis (92). Our results also showed that

both *EVC* and *METTL21C* were upregulated in L pigs. In contrast, an upregulation *EVC* and downregulation *METT21C*, and among other differentially expressed genes, were observed in *BRAF*-mutant cell lines, in response to metabolic stress through glucose withdrawal (93).

Conclusion

The high and low values of n-6/n-3 PUFA ratio on porcine skeletal muscle influence the expression profiles, related biological pathways and transcriptomic correlations and interactions between differentially expressed mRNAs and miRNAs. Predicted co-expression regulatory networks among mRNAs and miRNAs may be attributed to the pro- and anti-inflammatory functions of n-6 and n-3 PUFAs, respectively. Our findings highlighted mRNA genes, miRNAs and enriched pathways that were related to lipids metabolism, cell growth and inflammation, according to differences in muscle n-6/n-3 PUFA ratio.

Supporting Information

S1 Table. Phenotypic values of n-6/n-3 PUFA ratio, sex classification, and batch grouping recorded in 20 Iberian x Duroc pigs.

S2 Table. Differentially expressed genes (DEGs) according to n-6/n-3 PUFA ratio.

S3 Table. Differentially expressed miRNAs (DEmiRNAs) according to n-6/n-3 PUFA ratio.

S4 Table. List of Gene Ontology (GO) terms related to DEGs according to n-6/n-3 PUFA ratio.

S5 Table. Predicted porcine mature miRNAs co-expression with DEGs.

S6 Table. *miR-15b* seed binding interrogation on the 3' UTR of DEGs.

S7 Table. *miR-30a* and *miR-30e* seed binding interrogation on the 3' UTR of DEGs.

S8 Table. *miR-7142* seed binding interrogation on the 3' UTR of DEGs.

S9 Table. Meaningful co-expression network between negatively correlated DEGs and DEmiRNAs according to n-6/n-3 PUFA ratio.

S10 Table. Meaningful mRNA-mRNA co-expression of *ARRDC3* gene and other DEGs according to n-6/n-3 PUFA ratio.

S11 Table. Meaningful mRNA-mRNA co-expression of *METTL21C* gene and other DEGs according to n-6/n-3 PUFA ratio

S12 Table. List of genes significantly correlated with *ARRDC3* and *METTL21C* and their related functions

Data availability

All RNA- and miRNA-Seq data are available at NCBI Sequence Read Archive (SRA) under Accession PRJNA882638 and SUB12133922, respectively.

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Author contributions

Conceptualization: YJYM, AS, and JMF; Methodology: YJYM, LCM, EMS, AC, and JMF; Software: YJYM, LCM, EMS, and JMF; Formal analysis: YJYM, AEC, and JMF; Investigation: YJYM, LCM, AEC, AC, and JMF; Resources: AS and JMF; Data Curation: YJYM, LCM, AEC, EMS, and JMF; Writing—original draft: YJYM; Writing—review and editing: YJYM, LCM, EMS, AS, and JMF; Visualization: YJYM; Supervision: AS and JMF; Project administration: AS and JMF; Funding acquisition: AS and JMF. All authors have read and agreed to the published version of the manuscript.

Competing interest

The authors have declared that no competing interests exist.

References

1. Butler G. Manipulating dietary PUFA in animal feed: implications for human health. Proc Nutr Soc. 2014 Feb 5;73(1):87–95.

2. Berná G, Oliveras-López MJ, Jurado-Ruíz E, Tejedo J, Bedoya F, Soria B, et al. Nutrigenetics and nutrigenomics insights into diabetes etiopathogenesis. Nutrients. 2014;6(11):5338–69.

3. Marín-García PJ, Llobat L. How does protein nutrition affect the epigenetic changes in pig? A review. Animals. 2021;11(2):1–17.

4. Bartel DP. Metazoan MicroRNAs. Cell. 2018 Mar;173(1):20–51.

5. Kozomara A, Birgaoanu M, Griffiths-Jones S. MiRBase: From microRNA sequences to function. Nucleic Acids Res. 2019;47(D1):D155–62.

6. Sharma A, Park J-E, Chai H-H, Jang G-W, Lee S-H, Lim D. Next generation sequencing in livestock species- A Review. J Anim Breed Genomics. 2017;1(1).

7. Food and Agriculture Organization (FAO). Meat Market Review - Overview of market and policy developments in 2021 [Internet]. Rome; 2022. Available from: https://www.fao.org/3/cc0984en/cc0984en.pdf

8. Xing K, Zhao X, Ao H, Chen S, Yang T, Tan Z, et al. Transcriptome analysis of miRNA and mRNA in the livers of pigs with highly diverged backfat thickness. Sci Rep. 2019;9(1):1–12.

9. Martinsen KH, Ødegård J, Olsen D, Meuwissen THE. Genetic variation in efficiency to deposit fat and lean meat in Norwegian Landrace and Duroc pigs1. J Anim Sci. 2015 Aug 1;93(8):3794–800.

10. Rauw WM, Kanis E, Noordhuizen-Stassen EN, Grommers FJ. Undesirable side effects of selection for high production efficiency in farm animals: A review. Livest Prod Sci. 1998;56(1):15–33.

11. Patience JF. Strategies to maximize wean-to-finish net income: Focus on feed and

other technical issues. In: Ball RO, editor. Advances in Pork Production. Edmonton, AB: University of Alberta; 2010. p. 75–85.

12. National Research Council. Nutrient Requirements of Swine. 11th ed. Nutrient Requirements of Swine. Washington, D.C.: National Academies Press; 2012.

13. Bai L, Liang R, Yang Y, Hou X, Wang Z, Zhu S, et al. MicroRNA-21 regulates PI3K/Akt/mTOR signaling by targeting TGFβI during skeletal muscle development in pigs. PLoS One. 2015;10(5).

14. Sakayori N, Katakura M, Hamazaki K, Higuchi O, Fujii K, Fukabori R, et al. Maternal dietary imbalance between omega-6 and omega-3 fatty acids triggers the offspring's overeating in mice. Commun Biol. 2020 Dec 28;3(1):473.

15. Zivkovic AM, Telis N, German JB, Hammock BD. Dietary omega-3 fatty acids aid in the modulation of inflammation and metabolic health. Calif Agric. 2011 Jul;65(3):106–11.

16. Dennis EA, Norris PC. Eicosanoid storm in infection and inflammation. Nat Rev Immunol. 2015 Aug 3;15(8):511–23.

17. Martínez-Montes ÁM, Fernández A, Muñoz M, Noguera JL, Folch JM, Fernández AI. Using genome wide association studies to identify common QTL regions in three different genetic backgrounds based on Iberian pig breed. PLoS One. 2018;13(3).

18. Crespo-Piazuelo D, Criado-Mesas L, Revilla M, Castelló A, Noguera JL, Fernández AI, et al. Identification of strong candidate genes for backfat and intramuscular fatty acid composition in three crosses based on the Iberian pig. Sci Rep. 2020;10(1):1–17.

19. Andrews S. FastQC: A Quality Control Tool for High Throughput Sequence Data [Internet]. 2010. Available from: http://www.bioinformatics.babraham.ac.uk./projects/fastqc/

20. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing

reads. EMBnet.journal. 2011 May 2;17(1):10.

21. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013 Jan;29(1):15–21.

22. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011 Dec 4;12(1):323.

23. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10(3):R25.

24. Mármol-Sánchez E, Ramayo-Caldas Y, Quintanilla R, Cardoso TF, González-Prendes R, Tibau J, et al. Co-expression network analysis predicts a key role of microRNAs in the adaptation of the porcine skeletal muscle to nutrient supply. J Anim Sci Biotechnol. 2020 Dec 17;11(1):10.

25. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015 Jan 15;31(2):166–9.

26. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014 Dec 5;15(12):550.

27. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res. 2003 Nov;13(11):2498–504.

28. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics. 2009 Apr 15;25(8):1091–3.

29. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Ser B. 1995 Jan;57(1):289–300.

30. Reverter A, Chan EKF. Combining partial correlation and an information theory approach to the reversed engineering of gene co-expression networks.

Bioinformatics. 2008 Nov;24(21):2491–7.

31. Watson-Haigh NS, Kadarmideen HN, Reverter A. PCIT: an R package for weighted gene co-expression networks based on partial correlation and information theory approaches. Bioinformatics. 2010 Feb;26(3):411–3.

32. Shen W, Le S, Li Y, Hu F. SeqKit: A Cross-Platform and Ultrafast Toolkit for FASTA/Q File Manipulation. Zou Q, editor. PLoS One. 2016 Oct;11(10):e0163962.

33. Mayya VK, Duchaine TF. On the availability of microRNA-induced silencing complexes, saturation of microRNA-binding sites and stoichiometry. Nucleic Acids Res. 2015 Sep;43(15):7556–65.

34. Paschos GK, Ibrahim S, Song W-L, Kunieda T, Grant G, Reyes TM, et al. Obesity in mice with adipocyte-specific deletion of clock component Arntl. Nat Med. 2012 Dec 11;18(12):1768–77.

35. Laufer BI, Hasegawa Y, Zhang Z, Hogrefe CE, Del Rosso LA, Haapanan L, et al. Multi-omic brain and behavioral correlates of cell-free fetal DNA methylation in macaque maternal obesity models. bioRxiv. 2021 Jan 1;2021.08.27.457952.

36. Liu R, Liu X, Bai X, Xiao C, Dong Y. Different expression of lipid metabolism-related genes in Shandong black cattle and Luxi cattle based on transcriptome analysis. Sci Rep. 2020 Dec 14;10(1):21915.

37. Li K, Li J, Gu J, Guo X, Gao T, Li D. The protective effect of polyunsaturated fatty acid intake during pregnancy against embryotoxicity of sodium valproate in mice. Food Funct. 2018;9(5):2634–43.

38. Hopiavuori BR, Agbaga M-P, Brush RS, Sullivan MT, Sonntag WE, Anderson RE. Regional changes in CNS and retinal glycerophospholipid profiles with age: a molecular blueprint. J Lipid Res. 2017 Apr;58(4):668–80.

39. Oishi Y, Manabe I, Tobe K, Ohsugi M, Kubota T, Fujiu K, et al. SUMOylation of Krüppel-like transcription factor 5 acts as a molecular switch in transcriptional

programs of lipid metabolism involving PPAR-δ. Nat Med. 2008 Jun 25;14(6):656–66.

40. Hayashi S, Manabe I, Suzuki Y, Relaix F, Oishi Y. Klf5 regulates muscle differentiation by directly targeting muscle-specific genes in cooperation with MyoD in mice. Elife. 2016 Oct 15;5.

41. Lanktree MB, Joy TR, Hegele RA. The Metabolic Syndrome. In: Genomic and Personalized Medicine. Elsevier; 2013. p. 1006–16.

42. Rovadoscki GA, Pertile SFN, Alvarenga AB, Cesar ASM, Pértille F, Petrini J, et al. Estimates of genomic heritability and genome-wide association study for fatty acids profile in Santa Inês sheep. BMC Genomics. 2018 Dec 21;19(1):375.

43. Zhong L, Yang H, Zhu B, Zhao X, Xie M, Cao M, et al. The TBX1/miR-193a-3p/TGF-β2 Axis Mediates CHD by Promoting Ferroptosis. Daiber A, editor. Oxid Med Cell Longev. 2022 Jan 7;2022:1–13.

44. Yang WS, Kim KJ, Gaschler MM, Patel M, Shchepinov MS, Stockwell BR. Peroxidation of polyunsaturated fatty acids by lipoxygenases drives ferroptosis. Proc Natl Acad Sci. 2016 Aug 23;113(34).

45. Coyne GS, Kenny DA, Waters SM. Effect of dietary n-3 polyunsaturated fatty acid supplementation on bovine uterine endometrial and hepatic gene expression of the insulin-like growth factor system. Theriogenology. 2011 Feb;75(3):500–12.

46. Trivedi PC, Bartlett JJ, Mercer A, Slade L, Surette M, Ballabio A, et al. Loss of function of transcription factor EB remodels lipid metabolism and cell death pathways in the cardiomyocyte. Biochim Biophys Acta - Mol Basis Dis. 2020 Oct;1866(10):165832.

47. Jonscher KR, Alfonso-Garcia A, Suhalim JL, Orlicky DJ, Potma EO, Ferguson VL, et al. Spaceflight Activates Lipotoxic Pathways in Mouse Liver. Aspichueta P, editor. PLoS One. 2016 Apr 20;11(4):e0152877.

48. Würtz P, Wang Q, Soininen P, Kangas AJ, Fatemifar G, Tynkkynen T, et al.

Metabolomic Profiling of Statin Use and Genetic Inhibition of HMG-CoA Reductase. J Am Coll Cardiol. 2016 Mar;67(10):1200–10.

49. Karanth S, Tran VM, Kuberan B, Schlegel A. Polyunsaturated fatty acyl-coenzyme As are inhibitors of cholesterol biosynthesis in zebrafish and mice. Dis Model Mech. 2013 Jan 1;

50. Shimano H, Sato R. SREBP-regulated lipid metabolism: convergent physiology — divergent pathophysiology. Nat Rev Endocrinol. 2017 Dec 29;13(12):710–30.

51. DeBose-Boyd RA, Ye J. SREBPs in Lipid Metabolism, Insulin Signaling, and Beyond. Trends Biochem Sci. 2018 May;43(5):358–68.

52. Kolmus K, Tavernier J, Gerlo S. β2-Adrenergic receptors in immunity and inflammation: Stressing NF-κB. Brain Behav Immun. 2015 Mar;45:297–310.

53. Gorman AL, Dunn AJ. β-adrenergic receptors are involved in stress-related behavioral changes. Pharmacol Biochem Behav. 1993 May;45(1):1–7.

54. Gao M, Ping P, Post S, Insel PA, Tang R, Hammond HK. Increased expression of adenylylcyclase type VI proportionately increases β-adrenergic receptor-stimulated production of cAMP in neonatal rat cardiac myocytes. Proc Natl Acad Sci. 1998 Feb 3;95(3):1038–43.

55. Depre C, Ponchaut S, Deprez J, Maisin L, Hue L. Cyclic AMP suppresses the inhibition of glycolysis by alternative oxidizable substrates in the heart. J Clin Invest. 1998 Jan 15;101(2):390–7.

56. Nicolas C, Lacasa D, Giudicelli Y, Demarne Y, Agli B, Lecourtier M-J, et al. Dietary (n-6) Polyunsaturated Fatty Acids Affect β-Adrenergic Receptor Binding and Adenylate Cyclase Activity in Pig Adipocyte Plasma Membrane. J Nutr. 1991 Aug 1;121(8):1179–86.

57. Manaig Y, Sandrini S, Panseri S, Tedeschi G, Folch J, Sánchez A, et al. Low n-6/n-3 Gestation and Lactation Diets Influence Early Performance, Muscle and Adipose Polyunsaturated Fatty Acid Content and Deposition, and Relative

Abundance of Proteins in Suckling Piglets. Molecules. 2022 May 4;27(9):2925.

58. Mentzel CMJ, Alkan F, Keinicke H, Jacobsen MJ, Gorodkin J, Fredholm M, et al. Joint Profiling of miRNAs and mRNAs Reveals miRNA Mediated Gene Regulation in the Göttingen Minipig Obesity Model. Zheng Y, editor. PLoS One. 2016 Nov 30;11(11):e0167285.

59. Peng J, Zhao J-S, Shen Y-F, Mao H-G, Xu N-Y. MicroRNA Expression Profiling of Lactating Mammary Gland in Divergent Phenotype Swine Breeds. Int J Mol Sci [Internet]. 2015 Jan 8;16(1):1448–65. Available from: http://www.mdpi.com/1422-0067/16/1/1448

60. Bai Y, Huang J-M, Liu G, Zhang J-B, Wang J-Y, Liu C-K, et al. A comprehensive microRNA expression profile of the backfat tissue from castrated and intact full-sib pair male pigs. BMC Genomics. 2014 Dec 20;15(1):47.

61. Wang J, Hua L, Chen J, Zhang J, Bai X, Gao B, et al. Identification and characterization of long non-coding RNAs in subcutaneous adipose tissue from castrated and intact full-sib pair Huainan male pigs. BMC Genomics. 2017 Dec 19;18(1):542.

62. Sarkar S, Dey BK, Dutta A. MiR-322/424 and -503 are induced during muscle differentiation and promote cell cycle quiescence and differentiation by down-regulation of Cdc25A. Mol Biol Cell. 2010 Jul;21(13):2138–49.

63. Li X, Zhu L, Liu X, Sun X, Zhou Y, Lang Q, et al. Differential expression of microRNAs in porcine parvovirus infected porcine cell line. Virol J. 2015 Dec 20;12(1):128.

64. A. Hicks J, Yoo D, Liu H-C. Characterization of the microRNAome in Porcine Reproductive and Respiratory Syndrome Virus Infected Macrophages. Leung FCC, editor. PLoS One. 2013 Dec 5;8(12):e82054.

65. Jiang P, Zhou N, Chen X, Zhao X, Li D, Wang F, et al. Integrative analysis of differentially expressed microRNAs of pulmonary alveolar macrophages from

piglets during H1N1 swine influenza A virus infection. Sci Rep. 2015 Jul 2;5(1):8167.

66. Iikuni N, Kwan Lam Q, Lu L, Matarese G, Cava A. Leptin and Inflammation. Curr Immunol Rev. 2008 May 1;4(2):70–9.

67. Santoro N, Kursawe R, D'Adamo E, Dykas DJ, Zhang CK, Bale AE, et al. A common variant in the patatin-like phospholipase 3 gene (PNPLA3) is associated with fatty liver disease in obese children and adolescents. Hepatology. 2010 Aug 27;52(4):1281–90.

68. Corominas J, Marchesi JA, Puig-Oliveras A, Revilla M, Estellé J, Alves E, et al. Epigenetic regulation of the ELOVL6 gene is associated with a major QTL effect on fatty acid composition in pigs. Genet Sel Evol. 2015 Dec 25;47(1):20.

69. Jia H, Zhao Y, Li T, Zhang Y, Zhu D. miR-30e is negatively regulated by myostatin in skeletal muscle and is functionally related to fiber-type composition. Acta Biochim Biophys Sin (Shanghai). 2017 May;49(5):392–9.

70. Zaragosi L-E, Wdziekonski B, Brigand K Le, Villageois P, Mari B, Waldmann R, et al. Small RNA sequencing reveals miR-642a-3p as a novel adipocyte-specific microRNA and miR-30 as a key regulator of human adipogenesis. Genome Biol. 2011;12(7):R64.

71. Núñez-Hernández F, Pérez LJ, Muñoz M, Vera G, Accensi F, Sánchez A, et al. Differential expression of porcine microRNAs in African swine fever virus infected pigs: a proof-of-concept study. Virol J. 2017 Dec 17;14(1):198.

72. Ye L, Su X, Wu Z, Zheng X, Wang J, Zi C, et al. Analysis of Differential miRNA Expression in the Duodenum of Escherichia coli F18-Sensitive and -Resistant Weaned Piglets. Cascales E, editor. PLoS One. 2012 Aug 24;7(8):e43741.

73. Bao H, Kommadath A, Liang G, Sun X, Arantes AS, Tuggle CK, et al. Genome-wide whole blood microRNAome and transcriptome analyses reveal miRNA-mRNA regulated host response to foodborne pathogen Salmonella infection in

swine. Sci Rep. 2015 Oct 31;5(1):12620.

74. Kang JX, Liu A. The role of the tissue omega-6/omega-3 fatty acid ratio in regulating tumor angiogenesis. Cancer Metastasis Rev. 2013 Jun 23;32(1–2):201–10.

75. Kubiczkova L, Sedlarikova L, Hajek R, Sevcikova S. TGF-β – an excellent servant but a bad master. J Transl Med. 2012 Dec 3;10(1):183.

76. Wang W, Zhu J, Lyu F, Panigrahy D, Ferrara KW, Hammock B, et al. ω-3 Polyunsaturated fatty acids-derived lipid metabolites on angiogenesis, inflammation and cancer. Prostaglandins Other Lipid Mediat. 2014 Oct;113–115:13–20.

77. Podolska A, Anthon C, Bak M, Tommerup N, Skovgaard K, Heegaard PM, et al. Profiling microRNAs in lung tissue from pigs infected with Actinobacillus pleuropneumoniae. BMC Genomics. 2012 Dec 6;13(1):459.

78. Zhao Y, Rao J, Qiu T, Li C, Zhou X. The Effect of RBP4 on microRNA Expression Profiles in Porcine Granulosa Cells. Animals. 2021 May 13;11(5):1391.

79. Moore CAC, Milano SK, Benovic JL. Regulation of Receptor Trafficking by GRKs and Arrestins. Annu Rev Physiol. 2007 Mar 1;69(1):451–82.

80. Weinberg ZY, Puthenveedu MA. Regulation of G protein-coupled receptor signaling by plasma membrane organization and endocytosis. Traffic. 2019 Feb 11;20(2):121–9.

81. Nabhan JF, Pan H, Lu Q. Arrestin domain-containing protein 3 recruits the NEDD4 E3 ligase to mediate ubiquitination of the β2-adrenergic receptor. EMBO Rep. 2010 Aug 18;11(8):605–11.

82. Kang DS, Tian X, Benovic JL. Role of β-arrestins and arrestin domain-containing proteins in G protein-coupled receptor trafficking. Curr Opin Cell Biol. 2014 Apr;27:63–71.

83. Shea FF, Rowell JL, Li Y, Chang T-H, Alvarez CE. Mammalian Alpha Arrestins Link Activated Seven Transmembrane Receptors to Nedd4 Family E3 Ubiquitin Ligases and Interact with Beta Arrestins. Means RE, editor. PLoS One. 2012 Dec 7;7(12):e50557.

84. Patwari P, Emilsson V, Schadt EE, Chutkow WA, Lee S, Marsili A, et al. The Arrestin Domain-Containing 3 Protein Regulates Body Mass and Energy Expenditure. Cell Metab. 2011 Nov;14(5):671–83.

85. Ogawa M, Kanda T, Higuchi T, Takahashi H, Kaneko T, Matsumoto N, et al. Possible association of arrestin domain-containing protein 3 and progression of non-alcoholic fatty liver disease. Int J Med Sci. 2019;16(7):909–21.

86. Batista TM, Dagdeviren S, Carroll SH, Cai W, Melnik VY, Noh HL, et al. Arrestin domain-containing 3 (Arrdc3) modulates insulin action and glucose metabolism in liver. Proc Natl Acad Sci. 2020 Mar 24;117(12):6733–40.

87. Wang C, Arrington J, Ratliff AC, Chen J, Horton HE, Nie Y, et al. Methyltransferase-like 21c methylates and stabilizes the heat shock protein Hspa8 in type I myofibers in mice. J Biol Chem. 2019 Sep;294(37):13718–28.

88. Wiederstein JL, Nolte H, Günther S, Piller T, Baraldo M, Kostin S, et al. Skeletal Muscle-Specific Methyltransferase METTL21C Trimethylates p97 and Regulates Autophagy-Associated Protein Breakdown. Cell Rep. 2018 May;23(5):1342–56.

89. Olsen T, Lee S, Vinknes K, Norheim F, Hjorth M, Turner C, et al. Tissue Expression of Methyltransferases in Response to Acute and Long-term Exercise in Sedentary Men. Research Square [Preprint]; 2021. Available from: https://www.researchsquare.com/article/rs-143201/v1 doi: 10.21203/rs.3.rs-143201/v1,

90. Wilund KR. Is the anti-inflammatory effect of regular exercise responsible for reduced cardiovascular disease? Clin Sci. 2007 Jun 1;112(11):543–55.

91. Soung YH, Pruitt K, Chung J. Epigenetic silencing of ARRDC3 expression in

basal-like breast cancer cells. Sci Rep. 2015 May 24;4(1):3846.

92. Huang J, Hsu Y-H, Mo C, Abreu E, Kiel DP, Bonewald LF, et al. METTL21C Is a Potential Pleiotropic Gene for Osteoporosis and Sarcopenia Acting Through the Modulation of the NF-κB Signaling Pathway. J Bone Miner Res. 2014 Jul;29(7):1531–40.

93. Fernández KAMG. Targeting NRAS mutant melanomas through metabolic stress. Ph. D. Thesis. Universitat Autònoma de Barcelona; 2021. Available from: https://ddd.uab.cat/pub/tesis/2021/hdl_10803_673108/kamgf1de1.pdf

PAPER II

Low n-6/n-3 gestation and lactation diets influence early performance, muscle and adipose polyunsaturated fatty acid content and deposition, and relative abundance of proteins in suckling piglets

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Abstract

Elevated omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids (PUFAs) ratios in swine diets can potentially impose a higher risk of inflammatory and metabolic diseases in swine. A low ratio between the two omega PUFAs has beneficial effects on sows' and piglets' production performance and immunity status. At present, there are few studies on how sow nutrition directly affects the protein and fat deposition in suckling piglets. Two groups of sows were fed diets with high or low n-6/n-3 polyunsaturated ratios of 13:1 (SOY) and 4:1 (LIN), respectively, during gestation and lactation. Longissimus dorsi muscle and adipose tissue from newborn piglets, nourished only with sow's milk, were subjected to fatty acid profiling by gas chromatography–mass spectrometry (GC-MS) and to proteomics assays based on nano-liquid chromatography coupled to high-resolution tandem mass spectrometry (nLC-HRMS). Fatty acid profiles on both muscle and adipose tissues resembled the magnitude of the differences between fatty acid across diets. Proteomic analysis revealed overabundance of 4 muscle and 11 adipose tissue proteins in SOY compared to LIN in both piglet tissues. The detected overabundance of haptoglobin, an acute-phase protein, and the stimulation of protein-coding genes and proteins related to the innate immune response and acute inflammatory response could be associated with the pro-inflammatory role of n-6 PUFAs.

Keywords: piglets; longissimus dorsi; adipose tissue; PUFA; proteomics; omega-6; omega-3; inflammation; fat deposition

1. Introduction

Commercial swine feed is mostly cereal- and soybean meal-based, accompanied by plant and plant oil sources largely comprising omega-6 (n-6) polyunsaturated fatty acids (PUFAs). Consequently, this makes the amount of n-6 in feed mixes about 10 times higher than omega-3 (n-3) PUFAs [1]. Mammals, for example pigs, cannot incorporate double bonds in fatty acids beyond carbon 9 and 10 as they lack the required desaturase enzyme for their de novo synthesis; hence, essential fatty acids such as n-6 and n-3 should be supplemented in their diet [2]. As the trend for n-6 PUFAs in swine diets is growing, the continuous ingestion of n-6-enriched diets can cause an imbalance between the two PUFAs. As precursors of eicosanoids resulting in antagonistic inflammatory functions (anti- and pro-inflammatory, respectively), n-3 and n-6 PUFAs may induce substrate competition that can potentially affect metabolic health and inflammatory modulation [3,4]. Eicosanoids are bioactive lipid mediators synthesized through PUFA oxygenation, the majority of which are derived from n-6 PUFAs. Eicosanoid signaling has been proposed as a primary pro-inflammatory component of innate immunity and can control immune system activity [5,6].

Low dietary ratio between n-6 and n-3 (i.e., 4:1) can improve weaning weight, survival, weight gain, and influence the total n-3 polyunsaturated fatty acids found in colostrum and milk [7]. Previous human and pig studies on n-3 supplementation have shown reduced risk of cardiovascular diseases, obesity, and metabolic syndrome and diseases, and even beneficial effects on placental metabolism, inflammatory status, and lipid transfer [8–12]. Sow diet influences the accumulation of fatty acids by piglets through placental lipid transfer. Diets during gestation directly affect sow's milk and piglet's plasma fatty acid composition. This also indicates how maternal adipose tissue acts as the first depot of dietary fatty acids, which are then mobilized around farrowing and transferred to piglets [13]. In addition, evidence has shown how maternal dietary composition could possibly influence the availability and similarity of fatty acids in human placenta. Placental expression of G protein-coupled receptor 120 (GPR120, a decosahexaenoic acid receptor) has been correlated with adipocyte differentiation in neonatal fat [11,14]. A study on rats

has also shown on how the inclusion of n-3 PUFAs increased the growth of the fetus and placenta and reduced the oxidative degradation of lipids by increasing the expression of antioxidant enzymes in placental zones [15]. Proteomic approaches are now being used to assess intramuscular fat (IMF) deposition and pork quality. Pigs with high IMF revealed upregulated phosphatidylinositols and phosphatidylserines, whereas potential biomarkers such as α-actin, myosin-1, and myosin-4, were correlated with the degradation of myofibrillar proteins, indicating proteolysis and meat adhesiveness [16,17]. Unfortunately, none of these studies considered any proteomic approaches or elucidated the direct effects of gestating and lactating sow nutrition on the proteome of pre-weaning piglets. To our best knowledge, this work is one of the first studies to apply a proteomic approach in pre-weaned piglets fed with different n-6/n-3 ratio diets. The study was conducted to determine on how the milk of the sow, when fed with high or low n-6/n-3 PUFA ratio diets, directly affects and influences the fat deposition in muscle and in adipose tissues of pre-weaned piglets and the abundance of proteins, protein-coding genes, and their related ontologies and biological pathways.

2. Results

2.1. Sow Reproductive Performance

The overall reproductive performance of eight sows, four per treatment group, is summarized in Table 1. Dietary treatments had no effect ($p > 0.05$) on sow body weight, weight gain during gestation, or lactation periods. In addition, SOY and LIN did not affect the total number of piglets born (LSM ± SEM: SOY 15.5 ± 1.31 piglets; LIN 14.0 ± 1.31 piglets; $p > 0.05$), but subsequently, SOY increased the number of dead-born piglets compared to LIN (SOY 2.50 ± 0.41 piglets; LIN 0.50 ± 0.41 piglets; $p = 0.01$). The total piglets weaned per sow in the SOY group (12.00 ± 1.34 piglets) and the LIN group (12.50 ± 1.34 piglets) were similar ($p > 0.05$). Furthermore, no significant differences in pre-weaning mortality were found between SOY and LIN (22.50% ± 0.06% vs. 11.0% ± 0.06%, respectively; $p > 0.05$).

Table 1. Reproductive performance of sows between dietary treatments*.

Item	SOY	LIN	SEM	*p*-Value
Sow weight, kg				
insemination	266.00	259.50	12.75	0.73
d 30	271.56	254.50	9.17	0.24
d 60	283.88	269.38	8.51	0.27
farrowing	312.10	296.00	7.98	0.20
Sow weight gain, kg				
insemination to d 30	5.56	−4.88	5.31	0.21
d 30 to d 60	12.31	14.88	3.18	0.59
d 60 to farrowing	28.23	26.63	3.38	0.75
insemination to farrowing	46.10	36.63	7.53	0.41
Number of piglets				
total born	15.50	14.00	1.31	0.45
born dead	2.50 [a]	0.50 [b]	0.41	0.01
weaned	12.00	12.50	1.34	0.80
pre-weaning mortality, %	22.50%	11.00%	0.06	0.19

Data are least square means. Sows were fed diets with dietary treatments of n-6/n-3 polyunsaturated fatty acid ratios of 13:1 (SOY) and 4:1 (LIN). * Eight fourth parity sows; four in each treatment group. [a,b] Values within a row lacking a common superscript letter are significantly different ($p < 0.05$).

2.2. Growth Performance of Litter and Pre-Weaning Piglets

Piglet growth performance is reported in Table 2. Both litter weight (LW) and litter weight gain (LWG) were not influenced ($p > 0.05$) by the dietary treatments during lactation (data not shown). Higher individual body weight was detected in LIN piglets on day (d) 15 of lactation (SOY 4.75 ± 0.16 kg; LIN 5.30 ± 0.16 kg; $p = 0.02$), but no differences between LIN and SOY were observed at birth or weaning. As for piglet body weight gain (PBWG), improved performance was found in the first two weeks of life in

LIN (SOY 3.19 ± 0.14 kg; LIN 3.85 ± 0.14 kg; p = 0.0006), while higher PBWG was reported in SOY from d 15 up to weaning (SOY 2.72 ± 0.13 kg; LIN 2.22 ± 0.13 kg; p = 0.01). Over all the lactation period, similar PBWG was found in LIN and SOY groups of piglets (SOY 5.95 ± 0.20 kg; LIN 6.04 ± 0.20 kg; p = 0.75). The average daily gain (ADG) between SOY and LIN litters did not differ from birth to weaning, whereas LIN diet increased the ADG of individual piglets within its litter group (SOY 0.21 ± 0.01 kg; LIN 0.26 ± 0.01 kg; p = 0.0006). No differences were found in the ADG between SOY and LIN piglets from d 15 to weaning and in the overall weaning period.

Table 2. Growth performance of pre-weaned piglets *.

Item	***n***	**SOY**	**LIN**	**SEM**	***p*-Value**
Piglet weight, kg					
d 0	108	1.51	1.44	0.04	0.33
d 15	104	4.75 [b]	5.30 [a]	0.16	0.02
at weaning	98	7.52	7.47	0.22	0.89
Piglet weight gain, kg					
d 0–15	104	3.19 [b]	3.85 [a]	0.14	0.0006
d 15–weaning	98	2.72 [a]	2.22 [b]	0.13	0.01
d 0–weaning	98	5.95	6.04	0.20	0.75
Piglet ADG, kg					
d 0–15	104	0.21 [b]	0.26 [a]	0.01	0.0006
d 15–weaning	98	0.55	0.58	0.02	0.38
d 0–weaning	98	0.23	0.24	0.01	0.52

Data are least square means. Sows were fed diets with dietary treatments with n-6/n-3 polyunsaturated fatty acid ratios of 13:1 (SOY) and 4:1 (LIN). * Number of piglets varies due to accounted mortality before weaning; d 0: SOY 54 piglets, LIN 54 piglets; d 15: SOY 51 piglets, LIN 53 piglets; at weaning: SOY 48 piglets, LIN 50 piglets. [a,b] Values within a row lacking a common superscript letter are different (p < 0.05).

2.3. Fatty Acid Composition of Muscle and Adipose Tissue Samples

A lower n-6/n-3 ratio in sow diets was reflected in a lower n-6/n-3 muscle (SOY 16.45 ± 0.40, LIN 9.72 ± 0.40; $p < 0.0001$) and adipose tissue (SOY 14.70 ± 0.17; LIN 7.47 ± 0.17; $p < 0.0001$) content in piglets, mainly due to the increased relative proportion of n-3 PUFAs in both tissues (SOY 1.26 ± 0.05; LIN 2.12 ± 0.05; $p < 0.0001$ and SOY 1.07 ± 0.02; LIN 1.91 ± 0.02; $p < 0.0001$; respectively) (Figure 1b). The administration of a diet with a low n-6/n-3 ratio also led to a decrease in the n-6 content in the piglet's adipose tissue, but was not observed in muscle tissue. In the present trial, the total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and PUFAs content of neither muscle nor adipose tissues were influenced by the adapted dietary treatment of the sow (Figure 1a), but specific differences were found in these tissues for the major n-6 PUFAs (Figure 1c) and major n-3 PUFAs (Figure 1d). Supplementing gestating and lactating sows with a n-6/n-3 ratio in the diet did not affect the relative proportion of the major n-6 PUFAs in the muscle of the piglet, but led to lower proportions of linoleic acid (LA), gamma γ-linoleic acid (GLA), and arachidonic acid (AA) in piglet adipose tissue. The low rate of n-6/n-3 in the maternal diet caused a generalized increase in all the major n-3 PUFAs in both the muscle and adipose tissue of the piglets

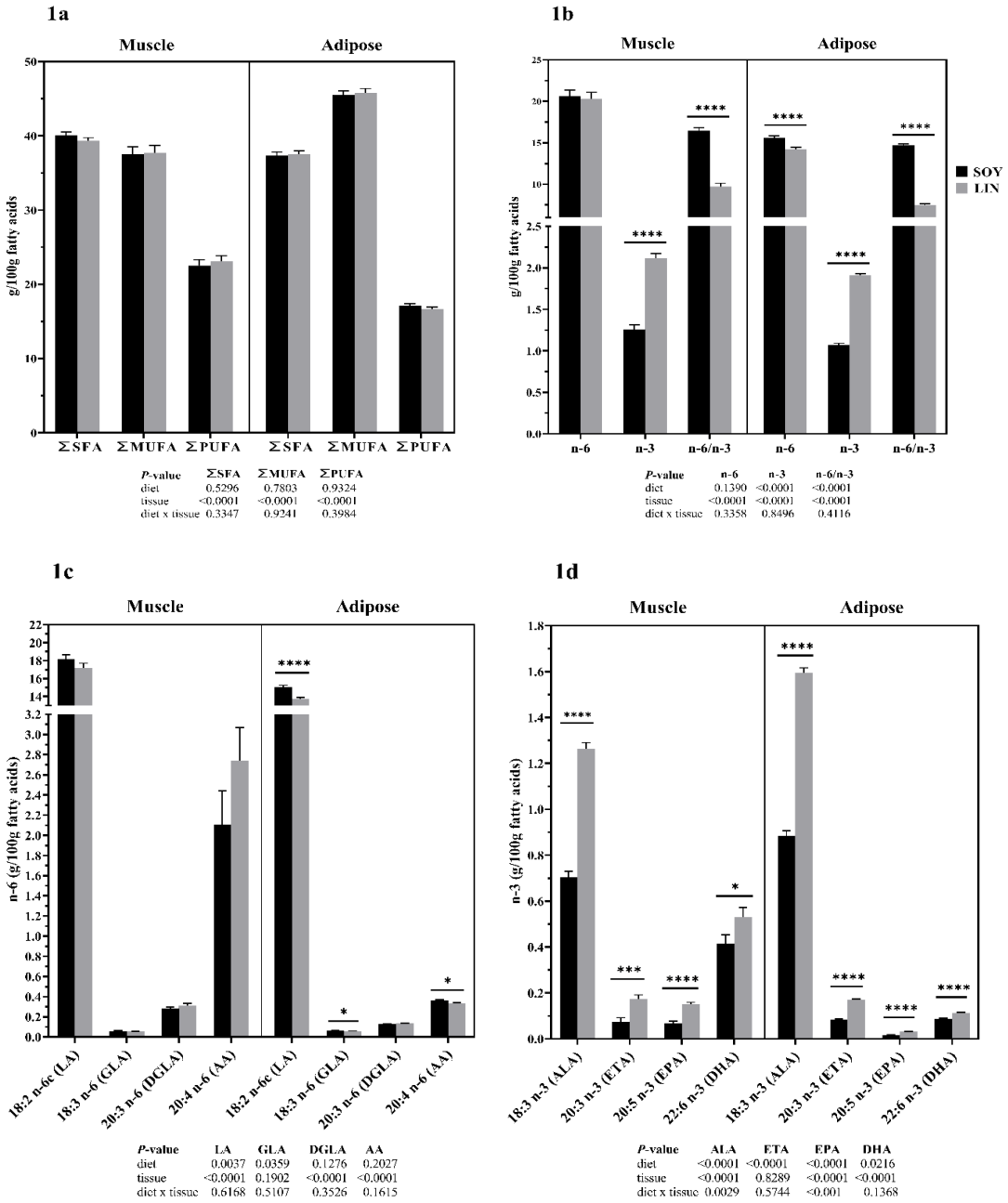

Figure 1. Fatty acid profile of muscle and adipose tissue samples for SOY and LIN diets. (**a**) Sum of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs); (**b**) total n-6, n-3, and the n-6/n-3 polyunsaturated fatty acid ratio; (**c**) major n-6 polyunsaturated fatty acid profile (linoleic acid, *LA*; gamma γ-linoleic acid, *GLA*; dihomo-gamma γ-linolenic acid, *DGLA*; arachidonic acid, *AA*); (**d**) major n-3 polyunsaturated fatty acid profile (alpha α-linolenic acid, *ALA*; eicosatrienoic acid, *ETA*; eicosapentaenoic acid, *EPA*; decosahexaenoic acid, *DHA*). Data are presented as LSM ± SE; significance levels: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = < 0.0001, no label = not significant, $p > 0.05$; $n = 24$ piglets for each SOY and LIN group.

2.4. Proteins Identification and Relative Abundance

2.4.1. From Muscle Tissues

A total of 339 proteins (Table S1) from the longissimus dorsi muscle were identified by Proteome Discoverer 2.5 software [18]. The identified muscle proteins were reported with their designated UNIPROT Accession number [19]. Abundance ratio comparison between dietary treatments SOY and LIN demonstrated four differentially expressed proteins ($P_{adj} < 0.05$), namely interferon-induced GTP-binding protein Mx2, prophenin and tritrpticin precursor, phosphoglycerate kinase 2, and haptoglobin. Moreover, three proteins—such as myoglobin, liver carboxylesterase, and protegrin-3—tended ($P_{adj} < 0.10$) to be differentially expressed (Table 3).

Table 3. Differentially expressed proteins* from muscle tissue, protein-coding genes, and their abundance ratio (SOY/LIN).

UNIPROT Accession	**Description**	**Gene**	**Abundance Ratio**	***P*$_{adj}$-Value**
A7VK00	Interferon-induced GTP-binding protein Mx2	*MX2*	100.00	6.50×10^{-16}
P51524	Prophenin and tritrpticin precursor	-	100.00	6.50×10^{-16}
Q6RI85	Phosphoglycerate kinase 2	*PGK2*	5.12	1.73×10^{-8}
Q8SPS7	Haptoglobin	*HP*	2.45	0.01
P02189	Myoglobin	*MB*	2.10	0.06
Q29550	Liver carboxylesterase	*CES1*	2.02	0.09
P32196	Protegrin-3	*NPG3*	2.00	9.55×10^{-2}

* Out of the 339 proteins identified, only overabundant proteins with P_{adj} value < 0.05 (significant) and < 0.10 (tendency) are shown.

2.4.2. From Adipose Tissue

Proteome Discoverer 2.5 software identified a total of 389 proteins (Table S2) from adipose tissue. Applying the same abundance ratio comparison (SOY vs. LIN), we obtained a total of 11 differentially expressed proteins ($P_{adj} < 0.05$)—60S ribosomal protein L29, myozenin-1, myosin light chain 4, myosin-4, haptoglobin, protegrin-2, liver carboxylesterase, phosphoglycerate kinase 2, histone H1t, glutathione S-transferase alpha M14, and desmoglein-1. In addition, we also included those proteins that tendend ($P_{adj} < 0.10$) to be overabundant in SOY over LIN, namely 60S ribosomal protein L35, metallothionein-1D, sarcoplasmic/endoplasmic reticulum calcium ATPase 3, and inter-alpha-trypsin inhibitor heavy chain H4. Their accession number, abundance ratio, and the genes encoding the aforementioned proteins are shown in Table 4.

Table 4. Differentially expressed proteins* from adipose tissue, protein-coding genes, and their abundance ratio (SOY/LIN).

UNIPROT Accession	Description	Gene	Abundance Ratio	P_{adj}-Value
Q95281	60S ribosomal protein L29	*RPL29*	100.00	1.18×10^{-15}
Q4PS85	Myozenin-1	*MYOZ1*	100.00	1.18×10^{-15}
F1RRT2	Myosin light chain 4	*MYL4*	6.93	2.47×10^{-13}
Q9TV62	Myosin-4	*MYH4*	5.12	3.33×10^{-9}
Q8SPS7	Haptoglobin	*HP*	3.56	2.66×10^{-5}
P32195	Protegrin-2	*NPG2*	2.95	7.16×10^{-4}
Q29550	Liver carboxylesterase	*CES1*	0.51	0.01
Q6RI85	Phosphoglycerate kinase 2	*PGK2*	0.52	0.01
P06348	Histone H1t	*H1-6*	2.37	0.02
P51781	Glutathione S-transferase alpha M14	*GSTA1*	2.34	0.02
Q3BDI7	Desmoglein-1	*DSG1*	0.57	4.96×10^{-2}
Q29361	60S ribosomal protein L35	*RPL35*	2.15	0.06

P79377	Metallothionein-1D	*MT1D*	2.10	0.08
O77696	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	*ATP2A3*	2.09	0.08
P79263	Inter-alpha-trypsin inhibitor heavy chain H4	*ITIH4*	2.08	0.09

* Out of the 389 proteins identified, only overabundant proteins with P_{adj} value < 0.05 (significant) and <0.10 (tendency) were shown.

2.5. Gene Ontologies and Biological Pathways

Due to the limited number of differentially expressed proteins ($p < 0.05$), genes encoding the abovementioned proteins were further investigated individually to generate ontologies and enrichment analysis. According to the UniProt database, genes found in muscle samples were related to the innate immune response (HP, MX2, P51524), glycolysis and gluconeogenesis (PGK2), and the acute inflammatory response (HP). Furthermore, genes found in adipose tissue samples exhibited relationships to GO pathways associated with the innate immune system (DSG1, NPG2), atherosclerosis (GSTA2), calcium ion binding and motor activity (MYH4, MYL4), cellular processes (GSTA2, RPL29, MYOZ1, HP), fatty acyl and cholesterol ester metabolism (CES1), glycolysis and gluconeogenesis (PGK2), and the acute inflammatory response (HP).

3. Discussion

The direct effect of high (13:1) and low (4:1) n-6/n-3 PUFA ratios in the sow's diet was investigated on the reproductive performance of sows, growth performance of pre-weaning piglets and the fat deposition in muscle and in adipose tissues, given that the piglets were only nourished with sow's milk. Moreover, changes in protein abundance, protein-coding genes, and their ontologies were further examined using proteomics data obtained from piglet tissues.

In our trial, LIN did not increase the total number of piglets born or weaned, but significantly decreased the number of dead-born piglets. Different sow studies found that n-3 PUFAs were able to decrease piglet mortality and improve pre- and post-weaning growth rates. Moreover, n-3 supplementation in the maternal diet was reviewed, along with how this benefits sow reproduction and piglet performance [20].

Piglet birth weight did not differ among dietary treatments, whereas an increase on piglet BW at d 15 was observed in the LIN group. Increased n-3 in gestational diets tends to cause increased piglet birth weight [21]. A meta-analysis in human studies also substantiated this claim, whereby n-3 PUFAs addition alone improved infant birth weight and was thus correlated to increased concentrations of n-3 PUFAs DHA and EPA [22]. Piglets from the SOY group had increased weight gain from d 15 to weaning. This can be attributed to the high AA concentration from the maternal diet, as major n-6 PUFAs (i.e., AA) have shown a positive correlation to neonatal growth. The association of AA content in piglet tissues and low birth weights was linked to the supplementation of n-3 PUFA in sow diets. The variability of the results on piglet growth performance may be related to substrate competition that can occur between the two PUFAs since n-3 and n-6 both serve as eicosanoid precursors and their inflammatory functions, synthesis, and oxidation pathways contrast each other [20,21,23]. We observed that litter weight and litter weight gain did not differ across dietary treatments and across defined subperiods. Among the sow reproductive studies that were reviewed, litter weight at birth did not significantly change upon the addition of n-3 PUFAs [20]. Although piglet weight and weight gain varied in between n-6/n-3 PUFA ratios, weaning weight and overall weight gain did not differ among dietary treatments.

Fatty acid profile on muscle and adipose tissues showed similarity to the dietary treatments and the same trend for total n-3, n-6, and n-6/n-3 ratios. From our results, sows fed SOY diets (n-6/n-3 ratio of 13:1) produced piglet with muscle and adipose tissue n-6/n-3 ratio of 16.45:1 and 14.70:1, respectively, while piglets from sows fed LIN (n-6/n-3 ratio of 4:1) had an n-6/n-3 ratio of 9.72:1 for muscle tissue and 7.47:1 for adipose tissue. Dietary ALA intake is shown to increase the DHA levels in piglet brain and EPA concentrations in the liver and in the blood of growing pigs [24,25]. This could explain the

higher level of EPA and DHA levels with LIN compared to SOY, considering the high level of ALA in linseed oil. Furthermore, the biosynthesis of DHA in vertebrates is now widely accepted. It follows a pathway called the *Sprecher* pathway, in which it is achieved by two consecutive elongations from EPA to produce tetracosapentaenoic acid (TPA, 24:5n−3), which then undergoes a Δ6 desaturation to tetracosahexaenoic acid (THA, 24:6n−3), the latter being β-oxidized to DHA in peroxisomes [26]. Thus, n-3 and n-6 PUFAs act as both a precursor and inhibitor, as they share the same enzymes for the synthesis of long-chain PUFAs. Following the magnitude of the difference between the omega ratio content of the diets and how this was reflected in piglet tissue, a clear deposition pattern of fatty acid into the animal was shown. Dietary fat consumed by the pigs directly affect the fatty acid composition of the carcass. It is generally dependent and mimics the fatty acid composition of the diet [27,28]. During digestion, dietary fatty acids are minimally hydrogenated or remain unchanged. If the pigs are fed above the maintenance requirement, the efficiency of dietary fat utilization is 90% [29]. The same n-6/n-3 ratios were used in sow gestation and lactation diets as were implemented in this study and colostrum and milk samples were collected at d 7 and at the end of lactation period [7]. It was noted how the low n-6/n-3 ratio increased the level of n-3 PUFAs, especially ALA, and decreased n-6/n-3 ratios in colostrum and milk samples. They also concluded that there was interaction between sampling point and sow diet. These further supported how the FA profiles of colostrum and milk, given that the piglets used in this trial were only fed with sow's milk, were mirrored and then incorporated into the muscle and subcutaneous fat of suckling pigs. In response to dietary fat ingestion, de novo synthesis of fatty acids is inhibited in favor of the direct deposition of fatty acids in adipose tissue, in which 31–40% of dietary fat is transferred to carcass lipids and to the intramuscular fat of muscle tissues (i.e., longissimus dorsi, semimembranosus, biceps femoris, diaphragm, masseter), regardless of fat source [30–33].

The relative proportion of fatty acids found in muscles was significantly higher in percentages than in adipose tissue, whereas the differences among fatty acids within adipose tissues in two dietary treatments were more defined. The accumulation of body fat occurs through cell hyperplasia (increase in cell number) and hypertrophy (increase in cell

size) after birth. During the early life stages, adipocyte hyperplasic development is favored; developed lipocytes become the first fat depots, which are usually found in perirenal, mesenteric, and intermuscular fat. As the animal grows, they continue to accumulate and manufacture more fat cells in subcutaneous and intramuscular deposits [34]. Hence, from a developmental point of view, intramuscular fat (IMF) is the last adipocytes depot in animals. Fat usually follows an order of deposition from perirenal fat, intermuscular fat, subcutaneous fat, and finally, through to intramuscular fat. We may hypothesize that the adipocytes found in subcutaneous fat have already reached their maximum storage capacity which, in turn, deposited all the excess available fat in the muscle tissue, thus increasing the amount of fat stored in the longissimus dorsi muscle. Furthermore, the IMF accumulation in muscle are dependent on the number and metabolic activity of adipocytes, growth rate of muscle tissues, and the metabolic activity of organs, such as the liver [35]. The longissimus dorsi muscle normally contains 1% of the total lipid found in pigs [36]. The age (d 26) of the piglets upon the collection of biological samples should also be taken into consideration, since subcutaneous carcass fat and total muscle lipid composition predominantly increase with the maturation of growing pigs [37]. Our results have also demonstrated how the relative proportion of n-6 PUFAs among dietary treatments resulted in more significant variations in adipose tissue than in muscle tissue, with the opposite for n-3 PUFAs. This may be due to high PUFA concentration of membrane lipids in the IMF, making them less vulnerable to nutritional modification [38].

The overabundance of muscle and adipose proteins found with SOY diets primarily indicated associations with pathways related to immune response, fatty acid metabolism, glycolysis and gluconeogenesis, and the inflammatory response. Due to contrasting function between n-6 and n-3 PUFAs, an increased supply of SOY in diets may trigger the pro-inflammatory function and effects of n-6 PUFA. The n-6/n-3 ratios of 9:1 and 13:1 have reportedly increased the immunoglobulin concentration in sow colostrum, although only 9:1 had effect on milk and in piglet plasma at d 21 of lactation. Although the mode of action of n-6 and n-3 PUFAs on immunoglobulins has not yet been elucidated, PUFAs are known to be involved in the production of white blood cell-derived cytokine called interleukins [39]. Eicosanoids are bioactive lipid mediators synthesized through

polyunsaturated fatty acid oxygenation. The majority of eicosanoids are produced from n-6 PUFA AA, while some arise from processing of n-3 PUFAs such as EPA or DHA. Eicosanoid signaling, as with cytokine signaling and inflammasome formation, has been associated as a primary pro-inflammatory component of innate immunity [5,6]. Although our results did not show any significant difference between the distribution of AA in muscle tissues, EPA was significantly and proportionally higher in adipose tissue. These two PUFAs act as competing substrates to cyclooxygenase (COX) and lipoxygenase (LOX) enzymes for eicosanoid synthesis [40]. The production of pro-inflammatory cytokines was further correlated to EPA/DHA:AA ratio in the membrane phospholipids of mononuclear cells, in human alveolar cells, and in smooth muscle cells [41–43]. Other pathways involving the overabundant proteins in adipose tissue are mostly related to fatty acid metabolism and processes and can be related to the addition of PUFAs in the diet.

Likewise, a total of four common overabundant proteins were found in both muscle and adipose tissue samples. These are haptoglobin, phosphoglycerate kinase 2, liver carboxylesterase, and porcine antimicrobial peptides protegrin (i.e., protegrin-2 and protegrin-3). The increased amount of haptoglobin in both muscle and adipose tissue could be related to the pro-inflammatory role of n-6 PUFAs. Animals that are subjected to health or stress-related challenges activate both their innate and acquired immune systems. The innate immune system involves the host defense mechanism, the production of antibodies, and leukocyte activity, whereas the acute-phase response is the biological reaction to infection, inflammation, or trauma. One of the pathophysiological responses is the plasma protein production, mainly in the liver, known as acute-phase proteins [44]. Haptoglobin is considered as one of the main acute-phase proteins in pigs and used as a diagnostic tool to assess diseases, health status, and production performance. As it is mostly synthesized in the liver and dependent on the synthesis of interleukin 6 (IL-6), it also protects the host against the dangers of acute-phase reactions [45–47]. In pigs, weaning is considered as a stressful event that can impact growth rate, gut health, immune functions, and nervous system functions that extends until maturity [48]. Serum levels of haptoglobin were elevated during weaning, which indicates exposure to stress or inflammatory stimuli [49]. In human studies, haptoglobin is considered as an indicator of obesity. It is expressed by

adipocytes and its abundance in white adipose tissue and in plasma shows a direct correlation with the degree of adiposity [50,51]. To our knowledge, this is the first study that detects haptoglobin in muscle and adipose tissue in pre-weaning piglets.

Increased levels of n-6 PUFA in the diet may have activated genes related to the glycolysis and gluconeogenesis pathway. *PGK2* encodes the protein that is responsible for the first catalytic ATP-generating step in the glycolytic pathway. Conversely, dietary PUFAs (i.e., LA, EPA, and DHA) are known potent inhibitors of metabolic enzymes and can suppress glycolytic and lipogenic genes [52,53]. Another common protein that was found is liver carboxylesterase. This enzyme is found in the liver and further protects the organ from alcohol- or diet-induced inflammation, damage, or injury [54]. Furthermore, SOY also stimulated an important class of cathelicidins known as protegrins. Cathelicidins are short cationic peptides that are part of the innate immune system for their antimicrobial capacity against Gram-positive and Gram-negative bacteria and their immunomodulatory functions [55,56].

We have also investigated the overabundant proteins and their protein-coding genes in response to SOY dietary treatment that were separately found in either muscle or adipose tissues. The increased detection of MX2, an interferon-stimulated gene induced by Type I interferons in response to viral infections, mainly HIV-1 [57], was only found in piglet muscle tissue. In humans, the PUFA AA has been shown to regulate the binding of interferon in skin fibroblast and n-6 PUFAs have been shown to influence its antiviral function [58,59]. Targeted lipodomics also revealed the correspondence of high levels of AA and low levels of EPA in phospholipid membranes to autoimmune diseases and how dietary supplementation of the n-3 PUFA DHA suppresses autoimmune pathogenesis and blocks gene expression pathways related to interferon [60,61]. Another protein-coding gene that was identified in adipose tissue is DSG1, an adhesive desmosomal protein that preserves human epidermis structure. A study in human squamous cell carcinoma revealed the inhibition of ETA on the expression of desmoglein and how the n-6 PUFA GLA upregulated its activity [62,63]. n-6 also influences the regulation of GSTA2, an enzyme that reduces lipid peroxidation products. Multiple studies reported that AA reduced the activity of GST alpha enzymes in zebrafish and inhibited the hepatic glutathione-S-

transferase in mice. On the other hand, DHA activates the glutathione antioxidant systems, preventing oxidative deterioration and associated negative sensory attributes in pork and poultry meat, and it is differentially expressed as a inflammatory response to atherosclerosis in mice [64–66]. Muscle and motor protein MYH4 was found to be up-regulated when pig adipose and skeletal muscle were exposed to glucose oxidation-promoting factor, such as cold exposure [67]. PUFA treatments (DHA, EPA, or AA) in muscle cell cultures also significantly decrease myosin heavy chain genes expression levels. By comparing the effect of DHA and AA supplementation on the same study, it could be concluded that n-3 has a greater inhibitory effect on myoblasts [68,69]. The results of the same study showed that DHA and EPA also downregulated MYL4, suggesting that different concentrations of PUFAs can also regulate mRNA expression levels in muscle and adipose tissues.

4. Materials and Methods

4.1. Experimental Design, Animals, and Housing

The research was conducted at the gestation and lactation facilities of Animal Production Research and Teaching Centre of the Department of Veterinary Medicine and Animal Science, Università degli Studi di Milano (Lodi, Italy). All experimental protocols were approved by the ethical committee of the University of Milan (OPBA 22/2020).

Sows were artificially inseminated with pooled semen according to heat cycle schedule. A total of 8 fourth parity sows (Topig40 × Topigs Fomeva, Topigs Norsvin, Vught, The Netherlands), with an average body weight of 256.56 ± 10.76 kg (mean ± SEM) and body condition score of 3.25 ± 0.89 (mean ± SEM), were selected after pregnancy confirmation by echographic assay performed on d 30 after artificial insemination and were used for the trial. Sows remained at gestation facilities until they were moved to individual farrowing crates on d 108 of gestation. Rooms were equipped with computer-controlled heating and mechanical ventilation systems and were monitored over a 24 h period. Initial room temperature was set at 28 °C with a ventilation rate of 10 m^3/h/head, and then

gradually decreased by 1 °C/week until to a final temperature of 25 °C at the end of the trial. Sows were then maintained in the farrowing creates until weaning at an average d 25.75 of lactation. The sows were reared on slatted floor with individual feeders and ad libitum access to water.

On newborn piglets, ear notching and tagging, and iron injection were performed within 24 h of birth. Piglets were nourished only with sow's milk. At the end of lactation, a total of 48 piglets (24 males and 24 females) were selected for fatty acid analysis from a pool of 98 piglets with an average body weight of 7.49 ± 0.16 kg (mean ± SEM). The piglets were further selected down to 24 piglets (12 males and 12 females) for proteomic analysis.

4.2. Experimental Diets

Sows were randomly divided between two dietary treatments with n-6/n-3 PUFA ratios of 13:1 (SOY) and 4:1 (LIN) during gestation and lactation. The n-6 and n-3 fatty acid supplementation was derived from soybean oil and linseed oil (Mazzoleni s.pa., Bergamo, Italy) and were both added to the basal diets (Table 5). Sow gestation and lactation basal diets were adjusted to attain final n-6/n-3 PUFA ratios of SOY and LIN, with the addition of soybean and linseed oil, throughout gestation and lactation. All experimental diets were formulated to be isonitrogenous and isocaloric and to meet or exceed the nutrient requirement for gestating and lactating sows [28]. Total fatty acid profile of oil and dietary treatments was presented on Table 6.

Feeding of sows followed the same protocol as described in [7]. Sows were given liquid feed by mixing the basal diets and water. The amount of soybean and linseed oils were calculated and added daily on top to achieve the previously described total n-6/n-3 PUFA ratios of SOY and LIN, for both gestation and lactation feeding plans. The gestational diet was given at 2.5 kg/d with 11 g/d of soybean or linseed oil from the day of insemination to d 59, at 2.7 kg/d with 13 g/d of soybean or linseed oil from d 60 to d 89, and 3 kg/d with 15 g/d of soybean or linseed oil from d 90 until before parturition. Sows

were fed per pen (2 sows/pen) with individual feed troughs. The lactation diet was fed at 0.5 kg/d on the day of farrowing (d 0 and then gradually increased to a maximum of 8 kg/d at weaning), with the corresponding amount of soybean and linseed oil to attain the total n-6/n-3 PUFA ratios. Sows were fed twice a day and given ad libitum access to water. Piglets were only nurtured with sow's milk throughout the entire lactation period.

Table 5. Composition of basal sow diets.

Item	**Gestation**	**Lactation**
Ingredients (g/kg as fed basis)		
Barley	200.13	201.93
Maize	150.00	161.00
Wheat	150.00	80.00
Wheat bran	160.00	150.00
Sunflower meal, 36% CP	52.00	50.00
Soybean hulls	50.00	49.00
Soybean meal, 48% CP	40.00	89.00
Maize germ meal	40.00	60.00
Biscuits	31.00	70.00
Animal fat, lard	-	34.60
Fish meal, 64% CP	-	20.00
Calcium carbonate	9.52	16.40
Calcium sulfate	5.00	-
Monocalcium phosphate	3.60	3.70
Vitamin premix *	3.00	3.00
L-Lysine	1.65	3.59
Sodium chloride	1.50	3.50
L-Threonine	1.08	1.31
Magnesium sulfate	1.00	1.00

Attapulgite **	-	1.00
Liquid choline	0.50	0.50
Methionine	0.02	0.37
Tryptophan	-	0.10
Composition (%DM)		
Crude protein	14.30	17.00
Crude fat	2.98	6.07
Crude fiber	7.23	6.59
Ash	5.46	5.90
Ca	0.68	0.85
p	0.65	0.60
Lysine	0.70	1.07
Methionine	0.25	0.34
Met + Cys	0.53	0.64
Digestible energy (kcal/kg)	2838.16	3200.63

* Providing (per kg of complete diet): vitamin A, 399×10^4 IU; vitamin D3, 650,000 IU; vitamin E, 50,000 mg; vitamin K3, 1355 mg; folic acid, 500 mg; niacinamide 10,000 mg; calcium pantothenate 7500 mg; vitamin B1 1000 mg; vitamin B2 2000 mg; vitamin B6 1000 mg; vitamin B12 10 mg; biotin 333 mg; Fe (as $FeSO_4$), 54,974 mg; Cu (as $CuSO_4$), 3869 mg; Cu (as copper chelate of hydroxy analogue of methionine), 425 mg; Cu (as cupric chelate of amino acids hydrate), 125 mg; Mn (as MnO), 14,736 mg; Mn (Mn chelate of amino acids hydrate, 250 mg; Zn (as ZnO), 15,287 mg; Zn (as Zn chelate of hydroxyl analogue of methionine), 1085 mg; Zn (as Zn chelate of amino acids hydrate), 615 mg; Se (as Na_2SeO_3), 77.9 mg; Selenomethionine from *Saccharomyces cerevisiae* NCYC R646, 22 mg; I (as $Ca(IO_3)_2$), 250 mg; citric acid, 20 mg; orthophosphoric acid, 22.5 mg; butylated hydroxyanisole (BHA), 20 mg; butylhydroxytoluene (BHT), 40 mg; 6-phytase (EC 3.1.3.26), 83,500 OTU; and endo-1,4-beta-xylanase (EC 3.2.1.8), 501,000 EPU. ** A magnesium aluminosilicate mineral clay being used as an absorbent.

Table 6. Fatty acids * (g/100 g total fatty acids) of dietary oils and sow diets.

		Dietary Oils		Sow Basal Diets		Dietary Treatments			
						Gestation		Lactation	
Lipid Name	**Common Name**	**Soybean**	**Linseed**	**Gestation**	**Lactation**	**SOY**	**LIN**	**SOY**	**LIN**
C6:0	Caproic acid	*n.d.*	*n.d.*	0.01	*n.d.*	0.01	0.02	*n.d.*	*n.d.*
C10:0	Capric acid	*n.d.*	0.02	0.06	0.09	0.05	0.04	0.06	0.07
C12:0	Lauric acid	*n.d.*	*n.d.*	0.27	0.46	0.23	0.25	0.42	0.41
C14:0	Myristic acid	0.05	0.05	0.41	1.23	0.35	0.36	1.11	1.13
C14:1	Myristoleic acid	*n.d.*	*n.d.*	0.02	0.01	0.02	0.02	0.01	0.01
C15:0	Pentadecylic acid	0.01	0.02	0.08	0.06	0.08	0.07	0.04	0.06
C16:0	Palmitic acid	10.37	5.99	16.72	21.93	15.82	15.2	20.81	20.39
C16:1	Palmitoleic acid	0.06	0.07	0.24	1.2	0.22	0.2	1.09	1.07
C17:0	Margaric acid	0.08	0.05	0.12	0.17	0.12	0.11	0.14	0.16
C17:1	Heptadecenoic acid	0.03	0.03	0.05	0.03	0.04	0.04	0.02	0.01
C18:0	Stearic acid	5.04	3.98	3.84	8.46	4.01	3.86	8.13	8.03
C18:1 n-9 *trans*	Elaidic acid	0.03	0.01	0.08	0.04	0.08	0.07	0.04	0.05
C18:1 n-9 *cis*	Oleic acid	23.29	16.57	24.25	33.49	24.11	23.16	32.5	31.84
C18:2 n-6 *cis*	Linoleic acid	52.46	16.3	49.01	29.58	49.5	44.37	31.8	28.29
C20:0	Arachidic acid	0.41	0.13	0.27	0.23	0.29	0.25	0.25	0.23
C18:3 n-6	Gamma-linoleic acid	0.01	0.22	0.01	0.01	0.01	0.04	0.03	0.04
C20:1 n-9	Eicosenoic acid	0.16	*n.d.*	0.38	0.57	0.35	0.33	0.53	0.51
C18:3 n-3	Alpha-linolenic acid	7.38	56.24	3.36	1.75	3.93	10.86	2.29	7.04
C21:0	Heneicosylic acid	0.02	*n.d.*	0.03	0.02	0.03	0.02	0.03	0.01
C20:2	Eicosadienoic acid	0.02	0.03	0.05	0.26	0.05	0.04	0.24	0.22
C22:0	Behenic acid	0.39	0.1	0.21	0.1	0.23	0.21	0.12	0.1
C22:1 n-9	Erucic acid	*n.d.*	0.03	0.04	0.01	0.03	0.04	0.02	0.03
C20:4 n-3	Eicosatetraenoic acid	0.01	0.05	0.01	0.03	0.01	0.01	0.03	0.04
C20:4 n-6	Arachidonic acid	0.04	*n.d.*	0.03	0.06	0.02	0.03	0.06	0.05
C23:0 n-6	Dihomo-gamma-linoleic acid	*n.d.*	0.01	0.04	*n.d.*	0.03	0.03	*n.d.*	*n.d.*
C22:2	Docosadienoic acid	*n.d.*	*n.d.*	0.04	*n.d.*	0.05	0.04	*n.d.*	*n.d.*
C24:0	Lignoceric acid	0.14	0.09	0.2	0.08	0.2	0.18	0.1	0.08

C20:5 n-3	Eicosapentaenoic acid	0.02	0.01	0.12	0.11	0.1	0.1	0.11	0.1
C24:1 n-9	Nervonic acid	*n.d.*	0.01	0.03	0.02	0.02	0.03	0.02	0.03
Saturated fatty acids (SFAs)		16.5	10.45	22.27	32.83	21.46	20.61	31.22	30.66
Monounsaturated fatty acids (MUFAs)		23.57	16.71	25.09	35.37	24.87	23.89	34.23	33.56
Polyunsaturated Fatty Acids (PUFAs)		59.93	72.84	52.64	31.8	53.67	55.5	34.56	35.78
Omega-3 (n-3)		7.4	56.3	3.48	1.89	4.04	10.98	2.43	7.18
Omega-6 (n-6)		52.5	16.52	49.06	29.64	49.54	44.44	31.89	28.38
n-6/n-3 ratio		7.09	0.29	14.1	15.64	12.27	4.05	13.11	3.95

* *n.d.* = not detected.

4.3. Recording and Sampling

Body weights (BW) of sows were assessed at the time of insemination, d 30, d 60, before transferring to farrowing crates (d 108), and at the end of lactation. One sow from the SOY group had to be removed from the study due to lameness, which affected its reproductive performance.

Piglets born, born alive, and born dead (stillborn, mummified, crushed, and abnormal) were counted within 24 h postpartum to calculate the survival rate at birth. Piglets were weighed at 24 h postpartum, d 15, and at weaning to calculate average daily weight gain. Feed efficiency cannot be calculated precisely since the piglets were suckling from sows, although 'weigh–suckle–weigh' procedure was an option but would have imposed great stress on piglets due to repeated weighing [70]. Litter weights were calculated as the sum of the individual piglet weights per sow per treatment. Piglet and litter weight gains were also calculated and divided into 3 subperiods— (1) At birth (d 0) to d 15, (2) from d 15 to weaning, (3) overall weight gain from birth up to weaning. At the end of lactation, the longissimus dorsi muscle and subcutaneous fat tissues were collected in triplicate during slaughtering, in accordance with European Council Regulation (EC) N° 1099/2009 protocols. The collected samples were labeled and placed in cryovials, snap-frozen in liquid nitrogen, and stored at −80 °C until analyses.

4.4. Muscle and Adipose Tissue Samples Fatty Acid Analysis

Extraction and derivatization of fat from muscle and adipose tissue samples were performed according to established lab protocols [71,72]. Fatty acid methyl esters (FAMEs) were measured using ThermoQuest Trace GC 2000 gas chromatography (Thermo Scientific, Bremen, Germany) with a flame ionization detector (Restek, Pennsylvania, USA); nonadecanoic acid (C19:0; 10 mg/mL of hexane) was the as internal standard and nitrogen (N) was the carrier gas. FAMEs were separated by a fused silica capillary column (Rt-2560, 100 m × 0.25 mm × 0.25 μm) and followed the given program: 70 °C for 5 min; increased by 2 °C min^{-1} until 240 °C, with total chromatographic runtime of 120 min. Individual FAMEs were verified by comparing peak retention times with standard mixtures (Supelco 37 FAME Mix, Bellefonte, PA, USA) and pure standard methyl esters from Sigma-Aldrich (Saint Louis, MO, USA).

4.5. Proteomics Analysis

4.5.1. Protein Extraction, Processing, and Digestion

Approximately 10 mg of longissimus dorsi muscle tissues were homogenized using a Varispeed A581 v.220 Potter homogenizer (Orlando Valentini, Milan, Italy) in 400 uL of extraction buffer (8 M urea, 20 mM Hepes pH 8.0, with Protease inhibitor cocktail) at full speed for 1 min [73]. The homogenate was sonicated using an ultrasonic probe in bursts of 20–30 s and centrifuged at 13,200 rpm for 15 min at 18 °C to pellet the tissue debris. Following dilution of urea to 2 M with NH_4HCO_3, 40 μg of protein was reduced (5 mM DTT, 30 min, 55 °C), alkylated (15 mM iodoacetamide, 20 min room temperature in the dark), and digested with (protein:trypsin ratio 40:1, 37 °C, overnight). Digestion was blocked by acidification with 4 μL trifluoroacetic acid. Digested peptides were desalted with Zip-tip according to the manufacturer's instructions, dried with Speedvac (Thermo Fisher Scientific, MA, United States) and resuspended in 0.1% formic acid (FA) prior to MS analysis [74].

4.5.2. nano-LC-MS/MS Analysis

Nano-HPLC was performed on a Dionex Ultimate 3000 nano-LC system (Sunnyvale, CA, USA) coupled to an Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with nano-electrospray ion source. Peptide mixtures were loaded onto a Acclaim PepMap 100 − 100 μm × 2 cm C18 (Thermo Scientific) and separated on EASY-Spray column ES802A, 25 cm × 75 μm ID packed with Thermo Scientific Acclaim PepMap RSLC C18, 3 μm, 100 Å using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile 20/80, v/v) at a flow rate 0.300 μL/min. The temperature was set to 35 °C. Samples were injected in duplicate. One blank was run between samples to prevent sample carryover. MS spectra were collected over an m/z range of 375–1500 Da at 120,000 resolutions, operating in the data dependent mode, cycle time 3 s between master scans. HCD MS/MS spectra were acquired on an Orbitrap at resolution of 15,000 using a normalized collision energy of 35%, and an isolation window of 1.6 m/z. Dynamic exclusion was set to 60 s. Rejection of +1 and unassigned charge states were enabled.

4.5.3. Proteomics Data Processing

Thermo raw data were analyzed against a protein database using SEQUEST algorithm in Proteome Discoverer software version 2.5 (Thermo Scientific) for peptide/protein identification [18]. MS/MS spectra were searched against Uniprot KnowledgeBase (KB)/Swiss-Prot *Sus scrofa* database (sp_canonical TaxID = 9823) (v2021-03-31). The minimum peptide length was set to six amino acids and enzymatic digestion with trypsin was selected, with maximum 2 missed cleavages. A precursor mass tolerance of 8 ppm and fragment mass tolerance of 0.02 Da were used; Acetylation (N-Terminus), Met-loss (M) and Met-loss + Acetylation (M) were used as dynamic modifications at protein terminus. Carbamidomethylation (C) was used as a static modification.

A decoy database search was performed to determine the peptide false discovery rate (FDR) with percolator node. The false discovery rates (FDRs) at the protein and peptide level were set to 0.01 for highly confident peptide-spectrum matches and 0.05 for peptide-spectrum matches with moderate confidence. Both unique and razor peptides were selected for protein quantification. Potential contaminants were filtered out using the PD_contaminants_2015 database (# 298 sequences). The abundance ratio for each protein is calculated based on the signal intensity ratios of isotopic pairs of peptide ions detected in the mass spectrometry scans [75].

4.6. Statistical Analysis

Data relative to piglet body weight (BW) and fatty acids were analyzed using the general linear model (GLM) procedure in SAS Studio 3.8, on SAS OnDemand for Academics release 9.04.01M6P11072018 (accessed November 30, 2021; https://welcome.oda.sas.com/home; SAS Institute Inc., Cary, NC, USA). Figures were plotted using GraphPad Prism version 8.3.0. (GraphPad software, La Jolla, CA, USA). The statistical model considered the n-6/n-3 PUFA ratios as fixed effects and individual piglets as repeated effect. Least squares means were calculated for each independent variable. The main n-6 polyunsaturated fatty acids, namely linoleic acid (LA), γ-linoleic acid (GLA), dihomo-γ-linolenic acid (DGLA), and arachidonic acid (AA), were grouped and calculated, as well as the major n-3 polyunsaturated fatty acids, such as α-linolenic acid (ALA), eicosatetraenoic acid (ETA), eicosapentaenoic acid (EPA), and decosahexaenoic acid (DHA). Detected saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) were summed and analyzed. Finally, the total n-6 and n-3 PUFAs and the n-6/n-3 PUFA ratios were summarized. Main effects of diet and tissue, and the interactive effects between the diet and tissue across diets were also determined using Tukey adjustment for multiple comparisons. Data were presented as LSM (least square means) ± SEM (standard error of means) in both tables and figures. The α-level that was used to determine significance was 0.05.

Proteins from muscle and adipose tissues were identified and reported with their designated UNIPROT Accession number. Protein ratios are calculated using Proteome Discoverer software version 2.5 (Thermo Scientific), as the median of all possible pairwise peptide ratios calculated between replicates of all connected peptides using *t*-test pairwise ratio-based approach. Protein quantification was based on the label-free quantification (LFQ) in which the mean LFQ intensities as well as the standard deviation of this value were calculated for SOY and LIN. The fold changes in the level of the proteins were assessed by comparing the mean LFQ intensities among all experimental groups. A protein was considered to be differentially expressed if the difference was statistically significant ($p \leq 0.05$), the minimum fold change was ±2, and a minimum of two peptides were identified.

5. Conclusions

The high and low dietary ratios between n-6 and n-3 fatty acids implemented on gestating and lactating sow diets influenced the fatty acid concentrations in both muscle and adipose tissues of pre-weaned piglets, and stimulated proteins and protein-coding genes related to innate immune response and acute inflammatory response. These findings show how low n-6/n-3 PUFA maternal diet can directly affect the early growth performance and fat deposition of suckling piglets. Some evidence of positive changes in immune status of piglets were also outlined by the proteome approach on muscle and fat tissue when sows are fed low n-6/n-3 PUFAs diets, but further studies including liver and serum proteomics could complement these findings, together with a correlation analysis between serum immunological status and proteomic assays.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27092925/s1, Table S1. The abundance ratio of *longissimus dorsi* muscle proteins derived from suckling piglets; Table S2. The abundance ratio of adipose tissue proteins derived from suckling piglets.

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References

1. Gjerlaug-Enger, E.; Haug, A.; Gaarder, M.; Ljøkjel, K.; Stenseth, R.S.; Sigfridson, K.; Egelandsdal, B.; Saarem, K.; Berg, P. Pig feeds rich in rapeseed products and organic selenium increased omega-3 fatty acids and selenium in pork meat and backfat. *Food Sci. Nutr.* **2015**, *3*, 120–128. https://doi.org/10.1002/fsn3.182.

2. Webster, J. *Animal Husbandry Regained*, 1st ed.; Routledge: London, UK, 2012; https://doi.org/10.4324/9780203094228.

3. Sakayori, N.; Katakura, M.; Hamazaki, K.; Higuchi, O.; Fujii, K.; Fukabori, R.; Iguchi, Y.; Setogawa, S.; Takao, K.; Miyazawa, T.; et al. Maternal dietary imbalance between omega-6 and omega-3 fatty acids triggers the offspring's overeating in mice. *Commun. Biol.* **2020**, *3*, 473. https://doi.org/10.1038/s42003-020-01209-4.

4. Zivkovic, A.M.; Telis, N.; German, J.B.; Hammock, B.D. Dietary omega-3 fatty acids aid in the modulation of inflammation and metabolic health. *Calif. Agric.* **2011**, *65*, 106–111. https://doi.org/10.3733/ca.v065n03p106.

5. Dennis, E.A.; Norris, P.C. Eicosanoid storm in infection and inflammation. *Nat. Rev. Immunol.* **2015**, *15*, 511–523. https://doi.org/10.1038/nri3859.

6. Lone, A.M.; Taskén, K. Proinflammatory and immunoregulatory roles of eicosanoids in T-cells. *Front. Immunol.* **2013**, *4*, 1–15. https://doi.org/10.3389/fimmu.2013.00130.

7. Nguyen, T.X.; Agazzi, A.; Comi, M.; Bontempo, V.; Guido, I.; Panseri, S.; Sauerwein, H.; Eckersall, P.D.; Burchmore, R.; Savoini, G. Effects of low Ω6:Ω3 ratio in sow Diet and seaweed supplement in piglet diet on performance, colostrum and milk fatty acid profiles, and oxidative status. *Animals* **2020**, *10*, 2049. https://doi.org/10.3390/ani10112049.

8. Duan, Y.; Li, F.; Li, L.; Fan, J.; Sun, X.; Yin, Y. n-6:n-3 PUFA ratio is involved in regulating lipid metabolism and inflammation in pigs. *Br. J. Nutr.* **2014**, *111*, 445–451. https://doi.org/10.1017/S0007114513002584.

9. Szostak, A.; Ogłuszka, M.; te Pas, M.F.W.; Poławska, E.; Urbański, P.; Juszczuk-Kubiak, E.; Blicharski, T.; Pareek, C.S.; Dunkelberger, J.R.; Horbańczuk, J.O.; et al. Effect of a diet enriched with omega-6 and omega-3 fatty acids on the pig liver transcriptome. *Genes Nutr.* **2016**, *11*, 9. https://doi.org/10.1186/s12263-016-0517-4.

10. Jump, D.B. Fatty acid regulation of hepatic lipid metabolism. *Curr. Opin. Clin. Nutr. Metab. Care* **2011**, *14*, 115–120. https://doi.org/10.1097/MCO.0b013e328342991c.

11. Wada, Y.; Yoshida-Yamamoto, S.; Wada, Y.; Nakayama, M.; Mitsuda, N.; Kitajima, H. Trans fatty acid accumulation in the human placenta. *J. Mass Spectrom.* **2017**, *52*, 139–143. https://doi.org/10.1002/jms.3910.

12. Leghi, G.E.; Muhlhausler, B.S. The effect of n-3 LCPUFA supplementation on oxidative stress and inflammation in the placenta and maternal plasma during pregnancy. *Prostaglandins Leukot. Essent. Fat. Acids* **2016**, *113*, 33–39. https://doi.org/10.1016/j.plefa.2016.08.010.

13. Amusquivar, E.; Laws, J.; Clarke, L.; Herrera, E. Fatty acid composition of the maternal diet during the first or the second half of gestation influences the fatty acid composition of sows' milk and plasma, and plasma of their piglets. *Lipids* **2010**, *45*, 409–418. https://doi.org/10.1007/s11745-010-3415-2.

14. Díaz, M.; García, C.; Sebastiani, G.; De Zegher, F.; López-Bermejo, A.; Ibáñez, L. Placental and cord blood methylation of genes involved in energy homeostasis: Association with fetal growth and neonatal body composition. *Diabetes* **2017**, *66*, 779–784. https://doi.org/10.2337/db16-0776.

15. Jones, M.L.; Mark, P.J.; Mori, T.A.; Keelan, J.A.; Waddell, B.J. Maternal dietary omega-3 fatty acid supplementation reduces placental oxidative stress and

increases fetal and placental growth in the rat. *Biol. Reprod.* **2013**, *88*, 1–8. https://doi.org/10.1095/biolreprod.112.103754.

16. Zhou, J.; Zhang, Y.; Wu, J.; Qiao, M.; Xu, Z.; Peng, X.; Mei, S. Proteomic and lipidomic analyses reveal saturated fatty acids, phosphatidylinositol, phosphatidylserine, and associated proteins contributing to intramuscular fat deposition. *J. Proteom.* **2021**, *241*, 104235. https://doi.org/10.1016/j.jprot.2021.104235.

17. López-Pedrouso, M.; Lorenzo, J.M.; Gagaoua, M.; Franco, D. Application of proteomic technologies to assess the quality of raw pork and pork products: an overview from farm-to-fork. *Biology* **2020**, *9*, 393. https://doi.org/10.3390/biology9110393.

18. Orsburn, B.C. Proteome discoverer-a community enhanced data processing suite for protein informatics. *Proteomes* **2021**, *9*, 15. https://doi.org/10.3390/proteomes9010015.

19. Bateman, A.; Martin, M.J.; Orchard, S.; Magrane, M.; Agivetova, R.; Ahmad, S.; Alpi, E.; Bowler-Barnett, E.H.; Britto, R.; Bursteinas, B.; et al. UniProt: The universal protein knowledgebase in 2021. *Nucleic Acids Res.* **2021**, *49*, D480–D489. https://doi.org/10.1093/nar/gkaa1100.

20. Tanghe, S.; De Smet, S. Does sow reproduction and piglet performance benefit from the addition of n-3 polyunsaturated fatty acids to the maternal diet? *Vet. J.* **2013**, *197*, 560–569. https://doi.org/10.1016/j.tvjl.2013.03.051.

21. Rooke, J.; SinclaiR, A.G.; Ewen, M. Changes in piglet tissue composition at birth in response to increasing maternal intake of long-chain n -3 polyunsaturated fatty acids are non-linear. *Br. J. Nutr.* **2001**, *86*, 461–470. https://doi.org/10.1079/BJN2001422.

22. Li, G.; Chen, H.; Zhang, W.; Tong, Q.; Yan, Y. Effects of maternal omega-3 fatty acids supplementation during pregnancy/lactation on body composition of the

offspring: A systematic review and meta-analysis. *Clin. Nutr.* **2018**, *37*, 1462–1473. https://doi.org/10.1016/j.clnu.2017.08.002.

23. Schmitz, G.; Ecker, J. The opposing effects of n-3 and n-6 fatty acids. *Prog. Lipid Res.* **2008**, *47*, 147–155. https://doi.org/10.1016/j.plipres.2007.12.004.

24. Arbuckle, L.D.; Innis, S.M. Docosahexaenoic acid in developing brain and retina of piglets fed high or low α-linolenate formula with and without fish oil. *Lipids* **1992**, *27*, 89–93. https://doi.org/10.1007/BF02535805.

25. Smink, W.; Gerrits, W.J.J.; Gloaguen, M.; Ruiter, A.; Van Baal, J. Linoleic and α-linolenic acid as precursor and inhibitor for the synthesis of long-chain polyunsaturated fatty acids in liver and brain of growing pigs. *Animal* **2012**, *6*, 262–270. https://doi.org/10.1017/S1751731111001479.

26. Oboh, A.; Kabeya, N.; Carmona-Antoñanzas, G.; Castro, L.F.C.; Dick, J.R.; Tocher, D.R.; Monroig, O. Two alternative pathways for docosahexaenoic acid (DHA, 22:6n-3) biosynthesis are widespread among teleost fish. *Sci. Rep.* **2017**, *7*, 1–10. https://doi.org/10.1038/s41598-017-04288-2.

27. Mitchaothai, J.; Yuangklang, C.; Wittayakun, S.; Vasupen, K.; Wongsutthavas, S.; Srenanul, P.; Hovenier, R.; Everts, H.; Beynen, A.C. Effect of dietary fat type on meat quality and fatty acid composition of various tissues in growing–finishing swine. *Meat Sci.* **2007**, *76*, 95–101. https://doi.org/10.1016/j.meatsci.2006.10.017.

28. National Research Council. *Nutrient Requirements of Swine*, 11th ed.; National Academies Press: Washington, DC, USA, 2012; https://doi.org/10.17226/6016.

29. Freeman, C.P. Fat supplementation in animal production—Monogastric animals. *Proc. Nutr. Soc.* **1983**, *42*, 351–359. https://doi.org/10.1079/PNS19830038.

30. Farnworth, E.R.; Kramer, J.K.G. Fat metabolism in growing swine: a review. *Can. J. Anim. Sci.* **1987**, *67*, 301–318. https://doi.org/10.4141/cjas87-029.

31. Chilliard, Y. Dietary fat and adipose tissue metabolism in ruminants, pigs, and rodents: a review. *J. Dairy Sci.* **1993**, *76*, 3897–3931. https://doi.org/10.3168/jds.S0022-0302(93)77730-9.

32. Kloareg, M.; Noblet, J.; van Milgen, J. Deposition of dietary fatty acids, de novo synthesis and anatomical partitioning of fatty acids in finishing pigs. *Br. J. Nutr.* **2007**, *97*, 35–44. https://doi.org/10.1017/S0007114507205793.

33. Realini, C.E.; Duran-Montgé, P.; Lizardo, R.; Gispert, M.; Oliver, M.A.; Esteve-Garcia, E. Effect of source of dietary fat on pig performance, carcass characteristics and carcass fat content, distribution and fatty acid composition. *Meat Sci.* **2010**, *85*, 606–612. https://doi.org/10.1016/j.meatsci.2010.03.011.

34. Sainz, R.D.; Hasting, E. Simulation of the development of adipose tissue in beef cattle. In *Modelling Nutrient Utilization in Farm Animals*; CABI: Wallingford, UK, 2000; pp. 175–182. https://doi.org/10.1079/9780851994499.0175.

35. Hocquette, J.F.; Gondret, F.; Baéza, E.; Médale, F.; Jurie, C.; Pethick, D.W. Intramuscular fat content in meat-producing animals: development, genetic and nutritional control, and identification of putative markers. *Animal* **2010**, *4*, 303–319. https://doi.org/10.1017/S1751731109991091.

36. Enser, M.; Hallett, K.; Hewitt, B.; Fursey, G.A.J.; Wood, J.D. Fatty acid content and composition of english beef, lamb and pork at retail. *Meat Sci.* **1996**, *42*, 443–456. https://doi.org/10.1016/0309-1740(95)00037-2.

37. Kouba, M.; Enser, M.; Whittington, F.M.; Nute, G.R.; Wood, J.D. Effect of a high-linolenic acid diet on lipogenic enzyme activities, fatty acid composition, and meat quality in the growing pig1. *J. Anim. Sci.* **2003**, *81*, 1967–1979. https://doi.org/10.2527/2003.8181967x.

38. Corino, C.; Rossi, R.; Cannata, S.; Ratti, S. Effect of dietary linseed on the nutritional value and quality of pork and pork products: Systematic review and meta-analysis. *Meat Sci.* **2014**, *98*, 679–688. https://doi.org/10.1016/j.meatsci.2014.06.041.

39. Yao, W.; Li, J.; Wang, J.; Zhou, W.; Wang, Q.; Zhu, R.; Wang, F.; Thacker, P. Effects of dietary ratio of n-6 to n-3 polyunsaturated fatty acids on immunoglobulins, cytokines, fatty acid composition, and performance of lactating sows and suckling piglets. *J. Anim. Sci. Biotechnol.* **2012**, *3*, 1–8. https://doi.org/10.1186/2049-1891-3-43.

40. Jameel, F.; Agarwal, P.; Arshad, M.; Serajuddin, M. Omega-3 polyunsaturated fatty acids of fish and their role in cancerous cell lines: A review of in-vitro studies. *Fish. Aquat. Life* **2019**, *27*, 47–63. https://doi.org/10.2478/aopf-2019-0006.

41. Cotogni, P.; Muzio, G.; Trombetta, A.; Ranieri, V.M.; Canuto, R.A. Impact of the ω-3 to ω-6 polyunsaturated fatty acid ratio on cytokine release in human alveolar cells. *J. Parenter. Enter. Nutr.* **2011**, *35*, 114–121. https://doi.org/10.1177/0148607110372392.

42. Bousserouel, S.; Brouillet, A.; Béréziat, G.; Raymondjean, M.; Andréani, M. Different effects of n-6 and n-3 polyunsaturated fatty acids on the activation of rat smooth muscle cells by interleukin-1β. *J. Lipid Res.* **2003**, *44*, 601–611. https://doi.org/10.1194/jlr.M200092-JLR200.

43. Endres, S.; Ghorbani, R.; Kelley, V.E.; Georgilis, K.; Lonnemann, G.; Van Der Meer, J.W.M.; Cannon, J.G.; Rogers, T.S.; Klempner, M.S.; Weber, P.C.; et al. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *N. Engl. J. Med.* **1989**, *320*, 265–271.

44. Jain, S.; Gautam, V.; Naseem, S. Acute-phase proteins: As diagnostic tool. *J. Pharm. Bioallied Sci.* **2011**, *3*, 118. https://doi.org/10.4103/0975-7406.76489.

45. Eurell, T.E.; Bane, D.P.; Hall, W.F.; Schaeffer, D.J. Serum haptoglobin concentration as an indicator of weight gain in pigs. *Can. J. Vet. Res.* **1992**, *56*, 6–9.

46. Petersen, H.H.; Ersbøll, A.K.; Jensen, C.S.; Nielsen, J.P. Serum-haptoglobin concentration in Danish slaughter pigs of different health status. *Prev. Vet. Med.* **2002**, *54*, 325–335. https://doi.org/10.1016/S0167-5877(02)00054-5.

47. Dobryszycka, W. Biological functions of haptoglobin-new pieces to an old puzzle. *Clin. Chem. Lab. Med.* **1997**, *35*, 647–654. https://doi.org/10.1515/cclm.1997.35.9.647.

48. Upadhaya, S.-D.; Kim, I.-H. The impact of weaning stress on gut health and the mechanistic aspects of several feed additives contributing to improved gut health function in weanling piglets—A review. *Animals* **2021**, *11*, 2418. https://doi.org/10.3390/ani11082418.

49. Sauerwein, H.; Schmitz, S.; Hiss, S. The acute phase protein haptoglobin and its relation to oxidative status in piglets undergoing weaning-induced stress. *Redox Rep.* **2005**, *10*, 295–302. https://doi.org/10.1179/135100005X83725.

50. Fain, J.N.; Bahouth, S.W.; Madan, A.K. Haptoglobin release by human adipose tissue in primary culture. *J. Lipid Res.* **2004**, *45*, 536–542. https://doi.org/10.1194/jlr.M300406-JLR200.

51. Maffei, M.; Barone, I.; Scabia, G.; Santini, F. The multifaceted haptoglobin in the context of adipose tissue and metabolism. *Endocr. Rev.* **2016**, *37*, 403–416. https://doi.org/10.1210/er.2016-1009.

52. Dentin, R. Polyunsaturated fatty acids suppress glycolytic and lipogenic genes through the inhibition of ChREBP nuclear protein translocation. *J. Clin. Invest.* **2005**, *115*, 2843–2854. https://doi.org/10.1172/JCI25256.

53. Yao, H.-T.; Chang, Y.-W.; Lan, S.-J.; Chen, C.-T.; Hsu, J.T.A.; Yeh, T.-K. The inhibitory effect of polyunsaturated fatty acids on human CYP enzymes. *Life Sci.* **2006**, *79*, 2432–2440. https://doi.org/10.1016/j.lfs.2006.08.016.

54. Xu, J.; Xu, Y.; Li, Y.; Jadhav, K.; You, M.; Yin, L.; Zhang, Y. Carboxylesterase-1 is regulated by hepatocyte nuclear factor 4α and protects against alcohol- and

MCD diet-induced liver injury. *Sci. Rep.* **2016**, *6*, 24277. https://doi.org/10.1038/srep24277.

55. Scheenstra, M.R.; van Harten, R.M.; Veldhuizen, E.J.A.; Haagsman, H.P.; Coorens, M. Cathelicidins modulate TLR-activation and inflammation. *Front. Immunol.* **2020**, *11*, 1–16. https://doi.org/10.3389/fimmu.2020.01137.

56. Harwig, S.S.L.; Waring, A.; Yang, H.J.; Cho, Y.; Tan, L.; Lehrer, R.I. Intramolecular disulfide bonds enhance the antimicrobial and lytic activities of protegrins at physiological sodium chloride concentrations. *Eur. J. Biochem.* **1996**, *240*, 352–357. https://doi.org/10.1111/j.1432-1033.1996.0352h.x.

57. Kane, M.; Yadav, S.S.; Bitzegeio, J.; Kutluay, S.B.; Zang, T.; Wilson, S.J.; Schoggins, J.W.; Rice, C.M.; Yamashita, M.; Hatziioannou, T.; et al. MX2 is an interferon-induced inhibitor of HIV-1 infection. *Nature* **2013**, *502*, 563–566. https://doi.org/10.1038/nature12653.

58. Nick, J.A.; Caceres, S.M.; Kret, J.E.; Poch, K.R.; Strand, M.; Faino, A.V.; Nichols, D.P.; Saavedra, M.T.; Taylor-Cousar, J.L.; Geraci, M.W.; et al. Extremes of interferon-stimulated gene expression associate with worse outcomes in the acute respiratory distress syndrome. *PLoS ONE* **2016**, *11*, e0162490. https://doi.org/10.1371/journal.pone.0162490.

59. Menon, S.D.; Yap, W.H.; Lim, A.; Tan, Y.H. Arachidonic acid regulates the binding of human interferon in human skin fibroblasts. *Lipids* **1990**, *25*, 321–327. https://doi.org/10.1007/BF02544341.

60. Rowbotham, R.; Duriancik, D. Eicosanoid metabolites associated with type-1 interferon production from plasmacytoid dendritic cells stimulated with toll-like receptors (P19-012-19). *Curr. Dev. Nutr.* **2019**, *3*, 1727. https://doi.org/10.1093/cdn/nzz049.P19-012-19.

61. Benninghoff, A.D.; Bates, M.A.; Chauhan, P.S.; Wierenga, K.A.; Gilley, K.N.; Holian, A.; Harkema, J.R.; Pestka, J.J. Docosahexaenoic acid consumption impedes early interferon- and chemokine-related gene expression while

suppressing silica-triggered flaring of murine lupus. *Front. Immunol.* **2019**, *10*, 1–25. https://doi.org/10.3389/fimmu.2019.02851.

62. Eynard, A.R.; Jiang, W.G.; Mansel, R.E. Eicosatrienoic acid (20:3 n-9) inhibits the expression of E-cadherin and desmoglein in human squamous cell carcinoma in vitro. *Prostaglandins Leukot. Essent. Fat. Acids* **1998**, *59*, 371–377. https://doi.org/10.1016/S0952-3278(98)90098-9.

63. Hammers, C.M.; Stanley, J.R. Desmoglein-1, differentiation, and disease. *J. Clin. Invest.* **2013**, *123*, 1419–1422. https://doi.org/10.1172/JCI69071.

64. Adam, A.-C.; Skjærven, K.H.; Whatmore, P.; Moren, M.; Lie, K.K. Parental high dietary arachidonic acid levels modulated the hepatic transcriptome of adult zebrafish (Danio rerio) progeny. *PLoS ONE* **2018**, *13*, e0201278. https://doi.org/10.1371/journal.pone.0201278.

65. Lee, S.A.; Whenham, N.; Bedford, M.R. Review on docosahexaenoic acid in poultry and swine nutrition: consequence of enriched animal products on performance and health characteristics. *Anim. Nutr.* **2019**, *5*, 11–21. https://doi.org/10.1016/j.aninu.2018.09.001.

66. Liu, Y.; Zhao, Y.; Shukha, Y.; Lu, H.; Wang, L.; Liu, Z.; Liu, C.; Zhao, Y.; Wang, H.; Zhao, G.; et al. Dysregulated oxalate metabolism is a driver and therapeutic target in atherosclerosis. *Cell Rep.* **2021**, *36*, 109420. https://doi.org/10.1016/j.celrep.2021.109420.

67. Xu, Z.; Chen, W.; Wang, L.; Zhou, Y.; Nong, Q.; Valencak, T.G.; Wang, Y.; Xie, J.; Shan, T. Cold exposure affects lipid metabolism, fatty acids composition and transcription in pig skeletal muscle. *Front. Physiol.* **2021**, *12*, 748801. https://doi.org/10.3389/fphys.2021.748801.

68. Zhang, J.; Xu, X.; Liu, Y.; Zhang, L.; Odle, J.; Lin, X.; Zhu, H.; Wang, X.; Liu, Y. EPA and DHA inhibit myogenesis and downregulate the expression of muscle-related genes in C2C12 myoblasts. *Genes* **2019**, *10*, 64. https://doi.org/10.3390/genes10010064.

69. Risha, M.A.; Siengdee, P.; Dannenberger, D.; Wimmers, K.; Ponsuksili, S. PUFA Treatment Affects C2C12 Myocyte Differentiation, Myogenesis Related Genes and Energy Metabolism. *Genes* **2021**, *12*, 192. https://doi.org/10.3390/genes12020192.

70. Noblet, J.; Etienne, M. Effect of energy level in lactating sows on yield and composition of milk and nutrient balance of piglets. *J. Anim. Sci.* **1986**, *63*, 1888–1896. https://doi.org/10.2527/jas1986.6361888x.

71. Folch, J.; Lees, M.; Stanley, G.H.S. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509. https://doi.org/10.1016/S0021-9258(18)64849-5.

72. Moltó-Puigmartí, C.; Castellote, A.I.; López-Sabater, M.C. Conjugated linoleic acid determination in human milk by fast-gas chromatography. *Anal. Chim. Acta* **2007**, *602*, 122–130. https://doi.org/10.1016/j.aca.2007.09.011.

73. Nonnis, S.; Maffioli, E.; Zanotti, L.; Santagata, F.; Negri, A.; Viola, A.; Elliman, S.; Tedeschi, G. Effect of fetal bovine serum in culture media on MS analysis of mesenchymal stromal cells secretome. *EuPA Open Proteom.* **2016**, *10*, 28–30. https://doi.org/10.1016/j.euprot.2016.01.005.

74. Mozzi, A.; Forcella, M.; Riva, A.; Difrancesco, C.; Molinari, F.; Martin, V.; Papini, N.; Bernasconi, B.; Nonnis, S.; Tedeschi, G.; et al. NEU3 activity enhances EGFR activation without affecting EGFR expression and acts on its sialylation levels. *Glycobiology* **2015**, *25*, 855–868. https://doi.org/10.1093/glycob/cwv026.

75. Griffin, T.J.; Lock, C.M.; Li, X.J.; Patel, A.; Chervetsova, I.; Lee, H.; Wright, M.E.; Ranish, J.A.; Chen, S.S.; Aebersold, R. Abundance-dependent proteomic analysis by mass spectrometry. In Proceedings of the 50th ASMS Conference on Mass Spectrometry and Allied Topics, Orlando, FL, USA, 2–6 June 2002; Volume 75, p. 51.

PAPER III

Exon-intron split analysis reveals post-transcriptional regulatory signals induced by high and low n-6/n-3 polyunsaturated fatty acid ratio diets in piglets

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Abstract

Polyunsaturated fatty acids (PUFAs), such as omega-6 (n-6) and omega-3 (n-3), play a vital role in nutrient metabolism, inflammatory response and gene regulation. Such regulation might be driven by microRNAs (miRNAs), which can potentially degrade targeted messenger RNAs and/or inhibit their translation. Although differential expression analyses can provide a comprehensive picture of gene expression variation, they are unable to disentangle when in the mRNA life cycle is regulation of expression taking place, including any putative functional miRNA-driven repression. In order to capture this, we used an exon-intron split analysis (EISA) approach to account for post-transcriptional changes in response to extreme values of n-6/n-3 PUFA ratio. *Longissimus dorsi* muscle samples of male and female piglets from sows fed with n-6/n-3 PUFA ratio of 13:1 (SOY) or 4:1 (LIN) were analyzed in a bidirectional contrast (LIN vs SOY, SOY vs LIN). Our results allowed the identification of genes showing strong post-transcriptional downregulation signals putatively targeted by significantly upregulated miRNAs. Moreover, we identified genes primarily involved in the regulation of lipid-related metabolism and immune responses, which may be associated to the pro- and anti-inflammatory functions of the n-6 and n-3 PUFAs, respectively. EISA allowed us to uncover regulatory networks complementing canonical differential expression analyses, thus providing a more comprehensive view of muscle metabolic changes in response to PUFA concentration.

Keywords: Exon-intron split analysis, PUFA, piglets, microRNA

1. Introduction

The landscape of post-transcriptional regulation of gene expression has been expanded significantly since the discovery of microRNAs (miRNAs), a class of regulatory small non-coding RNA molecules of ~22 nucleotides in length[1]. miRNAs have the ability to fine-tune the expression of genes linked to a specific metabolic pathway by base-pairing to the 3' untranslated region (3' UTR) of target messenger RNAs (mRNAs) and eliciting their degradation, thus potentially affecting the translation of tens or hundreds of mRNAs into functional proteins[2,3]. Besides, high-throughput transcriptome sequencing (RNA-Seq) has emerged as one of the primary methods in providing significant amount of data regarding gene expression profiles among multiple species, including pigs, and across diverse biological conditions[4–6]. One of the most popular applications of RNA-Seq data is differential expression (DE) analysis. This method enables the identification of genes that have different average expression levels between two or more conditions, which may aid to further explain some key phenotypic variations observed[7–9]. Although extremely helpful, utilizing DE analysis to infer gene regulation has a significant drawback – it does not account for transcriptional or post-transcriptional regulation that might alter gene expression estimates[10].

To circumvent this limitation, Gaidatzis *et al.*[11] developed the exon-intron split analysis (EISA), to disentangle the transcriptional and post-transcriptional components of gene regulation by measuring the changes between the exonic and intronic reads from expressed mRNA transcripts. The EISA approach considers the presence of reads mapping to intronic regions as being mainly derived from unprocessed mRNAs (pre-mRNAs) before splicing, hence indicative of transient transcriptional activation/repression in the nucleus. For modeling the post-transcriptional component of gene expression, EISA takes the difference between exonic and intronic changes, thus integrating the intronic fraction as an event happening before any post-transcriptional modification occurs in the cytoplasm[11]. Using the post-transcriptional signals determined by EISA and expression changes at the microRNA level, we can integrate both layers of information to link differentially expressed miRNAs and their targeted mRNAs at the post-transcriptional level.

Polyunsaturated fatty acids (PUFAs), particularly the omega-6 (n-6) and omega-3 (n-3) series, have the ability to influence and control gene expression and transcription factor activity, thus potentially affecting nutrient metabolism, regulatory networks, signal-transduction pathways, mRNA transcription, splicing, and protein synthesis[12,13]. Metabolites derived from n-6 and n-3 PUFAs are common precursors of eicosanoids that can regulate inflammation. As such, these two are also antagonistic in their inflammatory nature, with n-6 PUFAs acting as pro-inflammatory and n-3 PUFAs as anti-inflammatory modulators, respectively[14,15]. In livestock, maintaining a low ratio between n-6 and n-3 PUFAs on feeding diets has shown beneficial effects on reproductive and productive performance for both sow and piglets[16,17].

To better understand the effects of post-transcriptional regulation and PUFAs on mRNA expression and the miRNA regulatory contribution on porcine *longissimus dorsi* muscle, we determined post-transcriptional signals using EISA between male and female piglets from sows fed with high and low n-6/n-3 PUFA ratio diets. Moreover, we correlated both mRNA and miRNA expression profiles to the polyunsaturated fatty acids profile of skeletal muscle tissue.

2. Materials and Methods

2.1. Experimental design and animal material

Twenty-three (23) suckling piglets were obtained from a pool of piglets nourished only with both sow's colostrum and milk, as previously reported by Manaig *et al.*[17]. The gestating and lactating sows were divided between two treatments and fed *ad libitum* with diets containing n-6/n-3 PUFA ratios of 4:1 (**LIN**) or 13:1 (**SOY**). Such ratios were attained through the addition of soybean oil (n-6) or linseed oil (n-3) to the basal diet and maintained throughout the whole experimental period. At the end of lactation and before weaning, *longissimus dorsi* (LD) skeletal muscle samples were collected from selected piglets in triplicate immediately after slaughter, snapped-frozen, and stored at -80ºC. Fatty acids profiling of LD muscles was done using gas chromatography with a flame ionization

detector. A list of the measured fatty acids in the current study is available in **Additional Table 1a**. The selection of the 23 piglets included in this study was based on the analyzed values of n-6/n-3 PUFAs ratio for each treatment diet, i.e., 12 (LIN; 6 males, 6 females) and 11 (SOY; 6 males, 5 females) piglets were kept for further analyses. Recorded phenotypic values from the selected piglets are summarized at **Additional Table 1b**.

2.2. RNA extraction, library preparation and sequencing

Approximately 90 mg of LD tissue per sample was disrupted and homogenized in 700 µl of QIAzol Lysis Reagent (QIAGEN, Germantown, Maryland, USA) using 2 ml Lysing matrix D tubes (MP Biomedicals, Santa Ana, CA) and a Precellys 24 instrument (Bertin Technologies, Rockville, MD). The miRNeasy Kit (QIAGEN, Germantown, Maryland, USA) was used to extract the total RNA fraction while keeping small RNA molecules as per manufacturer's specifications. RNA was eluted with 30 µl of ultrapure water. RNA concentration and purity were determined with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Barcelona, Spain). RNA integrity was assessed with a Bioanalyzer-2100 equipment (Agilent Technologies Inc., Santa Clara, California, USA), using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Inc., Santa Clara, California, USA) and allowing an RNA Integrity Number (RIN) $\geq$ 7. Sequencing libraries were prepared with the TruSeq SBS Kit v3-HS (Illumina Inc., California, USA). A minimum of 30 million hits of 75 bp-length paired-end reads and a minimum of 10 million hits of 50 bp-length single-end reads were acquired per sample for mRNAs and small RNAs, respectively, using an Illumina HiSeq 3000/4000 equipment (CNAG-CRG, Barcelona, Centro Nacional de Análisis Genómico; https://www.cnag.crg.eu).

2.3. RNA-Seq and small RNA-Seq data processing

The FastQC[18] tool was used to perform quality control on raw fastq files from RNA-Seq and small RNA-seq sequences. Illumina adapters were trimmed using the Cutadapt v3.2[19] software. Only reads with 18-25 nucleotides (nts) in length after adapter trimming (compatible with the expected length of mature miRNA transcripts) were kept from small RNA-Seq data. Sequences from RNA-Seq data were mapped with HISAT2[20] aligner using default parameters, while small RNA-Seq data alignment was performed with the bowtie v1.2.1.1[21] tool, allowing no mismatches in the local mapping and a maximum of 20 multimapping spots per read, while reporting the best hit among the stacked multiple mappings (*bowtie -n 0 -m 20 -k 1 --best*)[22]. Both RNA-Seq and small RNA-Seq alignments were done against the reference pig genome Sscrofa11.1[23]. Dedicated annotations of exonic and intronic regions across the pig genome, excluding exonic-intronic site overlaps (10 bp at both ends of introns), were generated from all the annotated genes in the Sscrofa11.1 porcine reference assembly (Ensembl v106) by using the *makeEISAgtfs* function from the EISAcompR pipeline (https://github.com/emarmolsanchez/EISAcompR)[10]. Mature miRNA and mRNA expression levels, including the independent expression levels for exons and introns, were quantified using featureCounts v2.0.3[24] software.

2.4. Differential expression analyses

Differential expression analyses were carried out by using the exonic counts of mRNAs and the mature miRNA expression levels. We only considered genes with an average expression value above 1 count per million (CPM) in at least 50% of samples within each treatment group and sex classification – LIN male (**LIN-M**), LIN female (**LIN-F**), SOY male (**SOY-M**), and SOY female (**SOY-F**). Gene expression differences were tested separately for each sex, i.e. we compared male piglets fed with LIN diet (**LIN-M**) against male piglets fed with SOY diet (**SOY-M**), as well as female piglets fed with LIN diet (**LIN-F**) against female piglets fed with SOY diet (**SOY-M**). Raw counts were normalized for library size homogenization with the trimmed mean of M-values (TMM)[25]

method and the statistical significance of average expression differences between groups was assessed with a quasi-likelihood F-test (QLF) using edgeR[26] and including a batch correction in the regression model (**Additional Table 1b**). Multiple testing correction was performed with the false discovery rate approach[27]. mRNAs and mature miRNAs from DE analyses were considered significant at an absolute fold-change (FC) > 1.5 and *q*-value < 0.05. Suckling piglets from **LIN** diet (**LIN-M** and **LIN-F**) were defined as reference controls, meaning that any given gene upregulation (i.e., mRNAs or miRNAs) would imply an overexpression in **SOY**-fed piglets (**SOY-M** and **SOY-F**) with respect to **LIN**-fed piglets, leading to a positive fold change and vice versa.

2.5. Exon-intron split analysis

Post-transcriptional changes in messenger RNAs between diets were inferred using the EISAcompR pipeline (https://github.com/emarmolsanchez/EISAcompR)[10].

We only evaluated genes with both exonic and intronic read counts successfully quantified. The increase of intronic and exonic counts in **LIN-M** vs **SOY-M or LIN-F vs SOY-F** and, conversely, in **LIN-F** vs **SOY-F** represented the detected variations for every i^{th} gene. The increase in both the exonic and intronic fractions was calculated as follows: $\Delta Int = Int_{2i} - Int_{1i}$ for intronic counts; and $\Delta Ex = Ext_{2i} - Ex_{1i}$ for exonic counts, where Int_2 and Ex_2 represent each average i^{th} gene expression in the **SOY** diet for **LIN** vs **SOY**, or the average i^{th} gene expression in the **LIN** diet for **SOY** vs **LIN**, respectively. We then estimated the levels of post-transcriptional (PTc) change in mRNA expression. In this way, the difference between ΔEx and ΔInt represents the PTc component ($PTc = \Delta Ex - \Delta Int$)[28]. The top 5% of expressed genes with the most negative PTc scores were selected as genes displaying strong post-transcriptional regulatory signals. We also narrowed down our filtering criteria to those genes that had strongly reduced ΔEx showing at least 1.5-fold downregulation (*i.e.*, $\Delta Ex < -0.58$ in the $\log_2$ scale).

2.6. miRNA target prediction

Canonical target site prediction of the binding of miRNA seeds (2^{nd} to 8^{th} 5' nucleotide position in the mature miRNA) to the 3' UTR of target mRNA genes was performed by making use of the seedVicious v1.1[29] tool. The annotated 3' UTRs from porcine mRNAs were downloaded from Biomart database (http://www.ensembl.org/biomart, Sscrofa11.1 v106), while mature porcine miRNA sequences were obtained from miRBase v22.1[30] database. Only those mRNAs among the top 5% of expressed genes with the most negative PTc scores and at least 1.5-fold exonic (ΔEx) downregulation, as well as significantly upregulated miRNAs, were selected as input. MiRNA-mRNA interactions of type 8mer, 7mer-m8 and 7mer-A1 were considered. Furthermore, the expression correlation between the resulting miRNA-mRNA pairs was assessed by calculating Pearson's pairwise correlation coefficients (*r*) for each mRNA (exonic and intronic normalized $\log_2$ counts, separately) and miRNA (mature miRNA normalized $\log_2$ counts) from the pairs selected.

2.7. Gene ontology and pathway enrichment analysis

Differentially expressed mRNAs from exonic counts and those expressed genes with the top 5% most negative PTc scores and ΔEx repression of at least 1.5-fold among **LIN-M** vs **SOY-M**, **SOY-M** vs **LIN-M**, and **LIN-F** vs **SOY-F** contrasts were subjected to Gene ontology (GO) and pathway enrichment analyses. This was performed using Cytoscape v3.7.1 software with the ClueGO v2.5.4 plug-in application to determine enriched Biological Process terms[31,32]. Identification of enriched terms was done using a two-sided hypergeometric test of significance, with a false discovery rate approach for multiple testing correction[27].

2.8. Correlation between mRNAs, miRNAs and fatty acids profile of *longissimus dorsi* muscle

We estimated the Pearson's pairwise correlation coefficients (*r*) between normalized $\log_2$ expression values of genes that belong to the top 5% of expressed genes with the most negative PTc scores and at least 1.5-fold reduction in ΔEx values that were putatively targeted by upregulated miRNAs, differentially expressed miRNAs and the percent concentration of analyzed PUFA values of porcine LD muscle, as previously reported by Manaig *et al.*[17].

3. Results

3.1. Evaluation of post-transcriptional signals in *longissimus dorsi* muscle from male piglets

3.1.1. Differential expression and EISA

RNA-Seq data for male piglets obtained around 105 million reads per sample. From these, ~100 million reads (95%) per sample were successfully mapped to the Sscrofa11.1 reference assembly. Besides, around 9 million reads per sample were kept from small RNA-Seq data after length filtering (18-25 nts), from which ~5.8 million reads (65%) were successfully mapped to the 415 annotated porcine miRNA loci considered. The porcine reference assembly was then divided into its exonic and intronic regions to allow a split quantification of mapped reads from RNA-Seq data. Approximately 82% of the mapped reads from RNA-seq were assigned to exonic regions, while only 4% of the mapped reads overlapped intronic regions. With regard to small RNA-seq data, ~75% of the mapped reads between 18-25 nts were successfully assigned to porcine miRNAs.

With the help of edgeR software, we detected a total 11,055 porcine mRNAs as sufficiently expressed, 222 of which were differentially expressed with an absolute FC value greater than 1.5 and a *q*-value less than 0.05 (**Additional Table 2a**). From these, 174

genes were upregulated and 48 were downregulated in **SOY-M** pigs with respect to **LIN-M** pigs, as illustrated in **Fig. 1**. A full list of differentially expressed genes is available at **Additional Table 2b**. DE analyses on small RNA-Seq data highlighted a total of 62 significantly differentially expressed miRNAs – 29 downregulated and 33 upregulated miRNAs in **SOY-M** piglets with respect to **LIN-M** piglets (**Additional Table 3**).

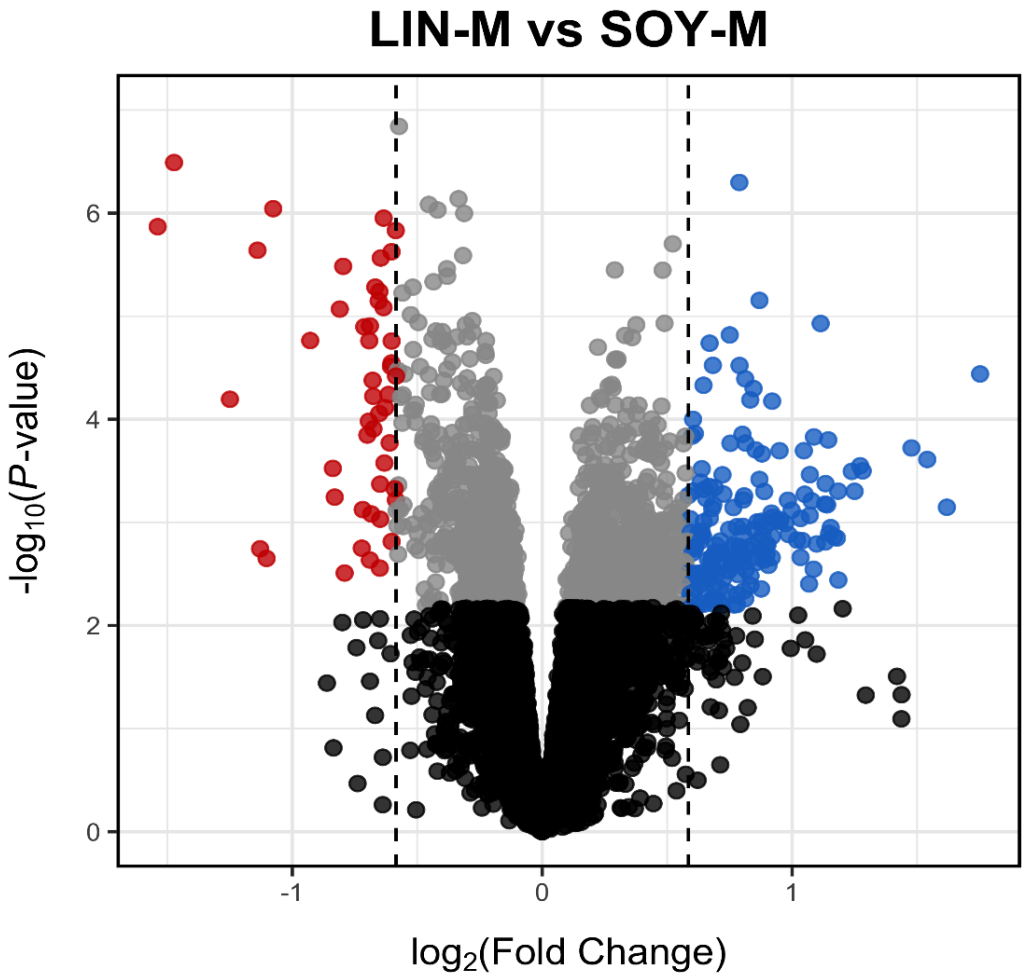

Figure 1. Volcano plot showing differentially expressed genes (|FC| > 1.5 and *q*-value < 0.05) in response to extreme n-6/n-3 PUFAs ratio in *longissimus dorsi* skeletal muscle. Genes with *P*-value > 0.05 are depicted in black. Upregulated genes (blue) correspond to genes overexpressed in **SOY-M** with respect to **LIN-M** piglets while downregulated genes (red) are those underexpressed in **SOY-M** compared to **LIN-M** piglets.

For the **LIN-M** vs **SOY-M** contrast, EISA revealed 11 genes within the top 5% negative PTc scores with at least 1.5-fold ΔEx reduction as shown in **Table 1**. Three out of these 11 genes (27.27%) were also reported to be significantly downregulated (FC > -1.5; *q*-value < 0.05) in **SOY-M** piglets according to canonical differential expression analyses (**Additional Table 2a**).

On the other hand, reversing our contrast to **SOY-M** vs **LIN-M** (**SOY-M** piglets are now considered as the reference group) allowed us to identify 97 mRNAs within the top 5% negative PTc scores and at least 1.5-fold ΔEx reduction in **LIN-M** piglets (summarized in **Additional Table 4)**. We also observed that out of these 97 genes, 22 (22.68%) of them were downregulated (FC > -1.5; *q*-value < 0.05) in **LIN-M** piglets after canonical DE analyses (**Additional Tables 2a** and **4**).

Table 1. Genes downregulated in **SOY-M** piglets with respect to **LIN-M** piglets and with the top 5% most negative post-transcriptional (PTc) scores and at least 1.5-fold exonic fraction (ΔEx) reduction from *longissimus dorsi* skeletal muscle samples.

Gene ID	Gene	ΔEx[a]	PTc[b]	DE[c]	miRNA target
ENSSSCG00000050235		-1.484	-5.827		
ENSSSCG00000027428	*ENHO*	-0.738	-4.625	×	×
ENSSSCG00000039271	*ST8SIA2*	-1.306	-4.558	×	×
ENSSSCG00000009114	*PRSS12*	-0.797	-3.208		×
ENSSSCG00000005938	*COL22A1*	-0.636	-3.053		×
ENSSSCG00000022868	*KCTD4*	-0.934	-2.898	×	×
ENSSSCG00000041170		-0.97	-2.413		
ENSSSCG00000003527	*EPHB2*	-0.679	-2.256		×
ENSSSCG00000008948	*ALB*	-0.738	-2.089		×
ENSSSCG00000036379		-1.477	-1.790		
ENSSSCG00000040349	*HOXD8*	-0.816	-1.676		×

[a]ΔEx, exonic fraction increment ($Ex_2 - Ex_1$) in the $\log_2$ scale when comparing exon abundances in **LIN-M** (Ex_1) vs. **SOY-M** (Ex_2) piglets.
[b]PTc, post-transcriptional signal (ΔEx − ΔInt) after z-score normalization.
[c]DE, significantly differentially expressed (|FC| > 1.5; *q*-value < 0.05).
The '×' symbol indicates significantly downregulated genes according to their exonic counts, as well as those mRNA genes targeted by at least one of the significantly upregulated miRNAs.

3.1.2. Target prediction between mRNAs with relevant PTc signals and upregulated miRNAs

We further selected the upregulated miRNAs and those mRNA genes with the top 5% most negative PTc and at least 1.5-fold exonic fraction reduction for the **LIN-M** vs **SOY-M** contrast for miRNA-mRNA target prediction (**Additional Table 5a**). Out of the 11 mRNA genes highlighted by EISA (**Table 1**), 8 of them (72.73%, **Table 1**) were targeted at least once by 24 out of the 33 (72.73%) upregulated miRNAs in **SOY-M** piglets with respect to **LIN-M** piglets (**Additional Table 5b**), providing a total of 34 8mer, 59 7mer-m8, and 43 7mer-A1 predicted miRNA binding sites (**Additional Table 5c**). The miRNA with the highest number of putative miRNA-mRNA interactions among the targeted mRNA genes was *ssc-miR-29b*, with six predicted interactions (**Additional Table 5b**). This was followed by *ssc-miR-214-5p* with five and *ssc-miR-142-5*, *ssc-miR-221-5p*, *ssc-miR-29a-3p*, *ssc-miR-204*, and *ssc-miR-195* with four interactions, respectively (**Additional Table 5b**). Besides, among this set of 8 targeted mRNAs, ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2 (*ST8SIA2*) showed the highest number of putative miRNA binding sites (13 out of 33; 39.39%), whereas both albumin (*ALB*) and serine protease 12 (*PRSS12*) showed 12 putative miRNA binding sites (36.36%) and homeobox D8 (*HOXD8*) reported a total of 9 (27.27%) (**Additional Table 5c**).

With regard to the reversed **SOY-M** vs **LIN-M** contrast (where we took **SOY-M** piglets as the reference group), 60 out of the 97 (61.86%) genes with the top 5% most negative PTc scores and displaying at least 1.5-fold ΔEx reduction in **LIN-M** piglets were targeted by at least one of the reported upregulated miRNAs in **LIN-M** piglets with respect to **SOY-M** piglets (**Additional Table 6a**). This set of 60 genes was targeted at least once by all 29 miRNAs upregulated in **LIN-M** piglets (and, conversely, downregulated in **SOY-M** piglets, **Additional Tables 4 and 6b**). Besides, a total of 95 8mer, 303 7mer-m8, and 280 7mer-A1 predicted putative miRNA binding sites were detected between the 60 targeted mRNA genes and the 29 upregulated miRNAs (**Additional Table 6c**). *Ssc-miR-532-3p* was the miRNA putatively targeting the highest number of mRNA genes (18 out of the 60, 30%), whereas *ssc-miR-885-3p* targeted 16 out of 60 genes (26.67%, **Additional**

Table 6b). The tripartite motif containing 14 (*TRIM14*) was the mRNA gene concentrating the highest number of putative binding sites for different upregulated miRNAs, totaling 18 out of 29, followed by transmembrane protein 71 (*TMEM71*) and von Willebrand factor A domain containing 5A (*VWA5A*) with both 15 miRNAs putatively targeting them, and interleukin-2 receptor subunit alpha (*IL2RA*) with 14 miRNAs, respectively (**Additional Table 6c**).

3.1.3. Exonic and intronic correlations of mRNA and miRNA genes

To further elucidate the miRNA post-transcriptional regulation, we calculated Pearson's pairwise correlation coefficients (r) between the normalized $\log_2$ expression values of upregulated miRNAs and the exonic and intronic fractions of their putative targeted mRNAs with high post-transcriptional signals. We then took the difference from exonic and intronic correlation values ($\Delta r = r_{ex} - r_{int}$) to assess the strength of correlation change in the exonic fraction with respect to the intronic fraction. Only those miRNA-mRNA pairs with putative predicted interactions were considered.

(1) **LIN-M** vs **SOY-M.** Based on the correlation values, the energy homeostasis associated (*ENHO*) gene displayed a complete switch in correlation values (Δr = -0.92) from positive intronic correlation (r_{int} = 0.03) to negative exonic correlation (r_{ex} = -0.89) compared to *ssc-miR-214-5p* expression (**Additional Table 7a**). This gene also demonstrated a stronger negative exonic correlation with two more miRNAs (*ssc-miR-29a-3p* and *ssc-miR-29b*). In addition, the ephrin type-B receptor 2 (*EPHB2*), *ST8SIA2*, and *HOXD8* showed a total reversal of correlation values to a few upregulated miRNAs such as *ssc-miR-23a*, *ssc-miR-221-5p*, *ssc-miR-218b*, respectively. A complete list of exonic, intronic, and change in correlation values of targeted genes and upregulated miRNAs for **LIN-M** vs **SOY-M** is shown in **Additional Table 7a**.

(2) **SOY-M vs LIN-M. Additional Table 7b** outlines the changes in correlation values from the intronic and exonic fractions between the predicted miRNA-mRNA pairs. The top 2 pairs that have the most negative change in correlation values were the

calcineurin like EF-hand protein 2 (*CHP2*) and *ssc-miR-532-5p,* as well as CTTNBP2 n-terminal like gene (*CTTNBP2NL*) and *ssc-miR-323,* with Δr at -0.99 and -0.97, respectively.

3.1.4. Functional analysis and pathway enrichment of differentially expressed genes

A total of 521 significant unique GO terms (q-value < 0.05) were detected from the differentially expressed genes related to **LIN-M** and **SOY-M** male piglets. Most of the significant GO terms were associated with immune response regulation and activation, along with carboxylic acid and polysaccharide metabolic and biosynthetic pathways. Some of these, to mention a few, are as follows: innate immune response (*GO:0045087*), regulation of cytokine-mediated signaling pathway (*GO:0001959*), regulation of alpha-beta T cell activation (*GO:0046634*), B cell differentiation (*GO:0030183*), positive regulation of interleukin-4 production (*GO:0032753*), unsaturated fatty acid biosynthetic process (*GO:0006636*), and glycogen metabolic process (*GO:0005977*). A full list of enriched GO terms is shown in **Additional Table 8**.

3.1.5. Association between fatty acids profile of longissimus dorsi muscle, PTc genes and upregulated miRNAs

We performed correlation analyses among genes with the top 5% negative PTc scores and at least 1.5-fold reduction in ΔEx values, upregulated miRNAs and the PUFA profiles of *longissimus dorsi* skeletal muscle (**Additional Table 1a**) for both **LIN-M** vs **SOY-M** and **SOY-M** vs **LIN-M** contrasts. Heatmap correlation plots were created to visualize paired correlations for (**1**) PUFAs vs mRNA genes (**Fig. 2a**), (**2**) PUFAs vs miRNAs (**Fig. 2b**), and (**3**) mRNA genes vs miRNAs (**Fig. 2c**). Likewise, heatmap plots for **LIN-M** piglets are shown in **Additional Figure 1a**, **1b**, and **1c**. Based on our results, both **SOY-M** and **LIN-M** displayed an overall negative correlation between the expression

of post-transcriptionally downregulated mRNAs and upregulated miRNAs, with the exception of collagen type XXII alpha 1 chain (*COL22A1*) and *ALB* mRNAs in **SOY-M** piglets (**Fig. 2c**) and lipase G (*LIPG*) and adiponectin (*ADIPOQ*) mRNAs in **LIN-M** piglets (**Additional Figure 1c**).

In **SOY-M** piglets, a clear positive correlation and clustering pattern was observed between individual n-3 PUFAs such as alpha-linolenic acid (C18:3 n-3, ALA) eicosatrienoic acid (C20:3 n-3, ETA), and eicosapentaenoic acid (C20:5 n-3, EPA), including the overall n-3 phenotype, and genes with negative PTc score, with the exception of *ALB* and *COL22A1* mRNAs, as shown in **Fig. 2a**. Moreover, we observed a negative correlation between most post-transcriptionally downregulated genes and the n-6 PUFAs, mainly driven by two n-6 PUFAs – linoleic acid (C18:2 n-6 *cis*, LA) and gamma-linoleic acid (C18:3 n-6, GLA). Similar anticorrelated patterns were also detected among PUFAs and upregulated miRNAs (**Fig. 2b**). In this case, while the same n-3 PUFAs (ALA, ETA and EPA) were mainly involved in the observed inverse relationship, gamma-linolenic acid (C18:3 n-6, GLA), dihomo-gamma-linoleic acid (C20:3 n-6, DGLA) and arachidonic acid (C20:4 n-6, AA) but not linoleic acid (C18:2 n-6 *cis*, LA) were the PUFAs driving the anticorrelation for the n-6 series (**Fig. 2b**).

Conversely, the correlation analyses for the relative proportions of PUFAs, as well as mRNA genes with strong post-transcriptional repression and upregulated miRNAs in **LIN-M** piglets were also summarized in **Additional Figure 1a**. All upregulated miRNAs in **LIN-M** piglets showed a clear negative correlation with post-transcriptionally downregulated mRNAs, except for *LIPG* and *ADIPOQ* (**Additional Figure 1c**). Besides, n-3 ALA, ETA and GLA fatty acids drove the observed negative correlation with mRNA genes, except for *LIPG*, while DGLA and AA but not LA and GLA fatty acids in the n-6 series also showed a clear anticorrelation with post-transcriptionally downregulated mRNAs and upregulated miRNAs in **LIN-M** piglets (**Additional Figure 1a and 1b**).

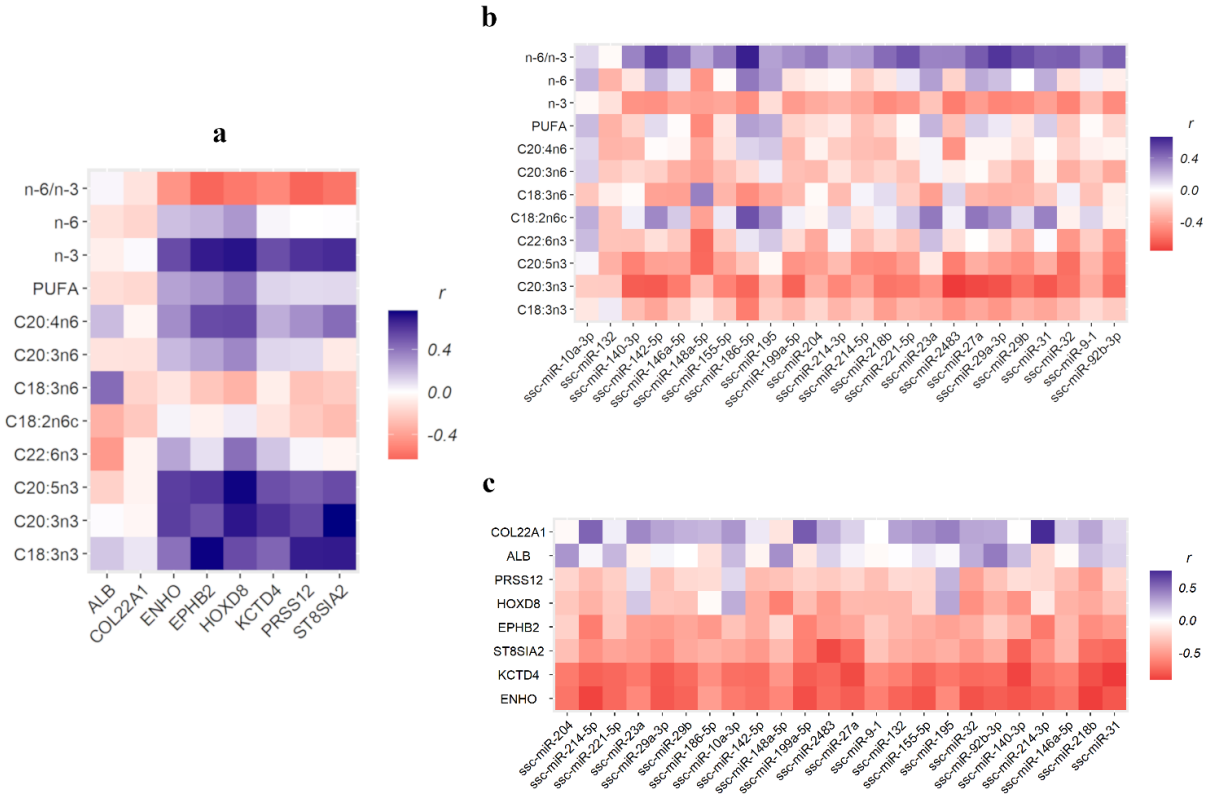

Figure 2. Heatmaps showing correlation values among mRNA genes with the top 5% negative PTc scores and at least 1.5-fold reduction in ΔEx values, upregulated miRNAs and PUFA profiles of *longissimus dorsi* skeletal muscle in **LIN-M** vs **SOY-M** piglets; (**a**) PUFAs vs mRNA genes; (**b**) PUFAs vs miRNAs; and (**c**) mRNA genes vs miRNAs.

3.2. Identifying post-transcriptional regulation in *longissimus dorsi* muscle from female piglets

RNA-Seq data for female piglets obtained around 100 million reads per sample. From these, ~97 million reads per sample were successfully mapped to the Sscrofa11.1 reference assembly. Besides, around 9.5 million reads per sample were kept from small RNA-Seq data after length filtering (18-25 nts), from which ~5.9 million reads (62%) were successfully mapped to 415 annotated porcine miRNAs considered. Approximately 79% of the mapped reads from RNA-seq were assigned to exonic regions, while only around 3.7% of mapped reads overlapped intronic regions. With regard to small RNA-seq data,

~75% of the mapped reads between 18-25 nts were successfully assigned to porcine miRNAs.

Using edgeR, we obtained a total of 11,025 expressed genes based on their exonic fraction, and 33 of them were highlighted as differentially expressed with an absolute FC value greater than 1.5 and a *q*-value less than 0.05. The identified differentially expressed genes were comprised of only upregulated genes in **SOY-F** piglets with respect to **LIN-F** piglets (**Fig. 3**) and summarized in **Additional Table 9a.** A full list of exonic-based differential expression analysis in **SOY-F** piglets is shown in **Additional Table 9b**. Only one miRNA (ssc-miR-142-5p) was deemed to be differentially expressed and upregulated (FC > 1.5 and *q*-value < 0.05) in **SOY-F** piglets (**Additional Table 10**).

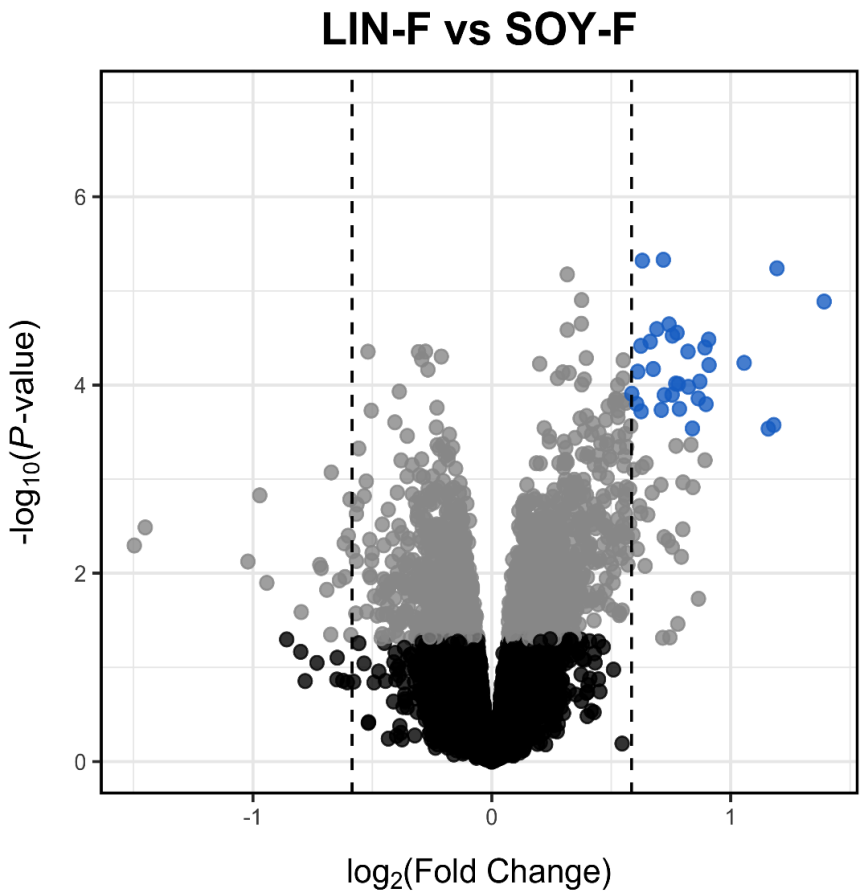

Figure 3. Volcano plot showing differentially expressed genes (fold change > |1.5| and p-adj value < 0.05) in response to extreme n-6/n-3 PUFAs ratio in *longissimus dorsi* skeletal muscle from **LIN-F** and **SOY-F** female piglets. Genes with *P*-value > 0.05 are depicted in black. Upregulated genes (blue) correspond to genes overexpressed in **SOY-F** group.

EISA highlighted 30 genes within the top 5% most negative PTc scores and at least 1.5-fold ΔEx reduction in **SOY-F** piglets, as shown in **Additional Table 11**. None of these genes were either upregulated or downregulated after canonical DE analyses. Furthermore, 6 out of the 30 (20%) genes were targeted by the upregulated miRNA *ssc-miR-142-5p*, totaling 7 7mer-m8 and 4 7mer-a1 putative binding sites (**Additional Table 12a** and **12b**). ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2 (*ST8SIA2*) was the mRNA gene with the highest number of 7mer-m8 and 7mer-A1 predicted binding sites for the upregulated miRNA *ssc-miR-142-5p.*

The post-transcriptional regulation between the targeted genes and the upregulated miRNAs was further illustrated using the change in correlation values from intronic to exonic regions (i.e., $\Delta r = r_{ex} - r_{int}$). Although most of the Δr were still negative in value, the intronic and exonic correlation between miRNA-mRNA pairs remained negative across both exonic and intronic fractions (**Additional Table 13**). Moreover, functional analysis and pathway enrichment analyses identified 15 significant unique GO terms (q-value < 0.05) from the exonic-based differentially expressed genes among female piglets (**Additional Table 14**). These biological pathways were mostly associated with regulation and activation of immune response such as T-cell mediated immunity (*GO:0002456*), positive regulation of interleukin-2 production (*GO:0032743*) and positive regulation of alpha-beta T cell activation (*GO:0046635*).

Correlation heatmaps for **LIN-F** vs **SOY-F** contrast were summarized in **Additional Figure 2a**, **2b**, and **2c**. Two of the analyzed n-3 PUFAs (i.e., ALA and ETA) demonstrated an overall positive correlation to all of the EISA-detected downregulated mRNA genes except for *PDZD9* (**Additional Figure 2a**), which revealed an overall clear negative correlation with most of the PUFAs. Moreover, all analyzed PUFAs except for ETA and n-6/n-3 PUFA ratio displayed a positive correlation with the upregulated *ssc-miR-142-5p* (**Additional Figure 2b**). In general, all the genes with most negative PTc values according to EISA showed a negative correlation with the overexpressed miRNAs in **SOY-F** piglets (**Additional Figure 2c**).

4. Discussion

4.1. Different set of genes between DE and exon-intron split analyses

Canonical differential expression analyses of exonic-based reads generally highlighted different sets of genes compared to those with strong post-transcriptional signals detected by EISA. Nevertheless, around 3 out of 11 (27.27%) of genes in **LIN-M vs SOY-M** and 22 out of 97 (22.68%) of genes in **SOY-M vs LIN-M** were shared between the two methodologies. Furthermore, the intronic and exonic correlation analysis between mRNA and miRNAs reads in **SOY-M vs LIN-M** (**Additional Table 7b**) established a more robust and clearer miRNA-driven post-transcriptional repression at the exon level compared to the more limited, yet strong regulatory signals elicited in **LIN-M vs SOY-M** contrast (**Additional Table 7a**). Such discrepancy could be attributed to the overall stronger and varied upregulation of miRNAs observed in **LIN-M** piglets.

Although no downregulated genes were detected in **LIN-F vs SOY-F** after canonical DE analyses, EISA was able to display 30 genes that belong to the top 5% negative PTc scores with an at least 1.5-fold ΔEx reduction (**Additional Table 11**). However, the post-transcriptional signals from female piglets were not as strong and clear as those from the males. The correlation values among miRNA-mRNA pairs remained negative both across intronic and exonic regions (**Additional Table 13**), indicating mixed transcriptional and post-transcriptional downregulation signals at play that might confound our analyses. Moreover, we obtained only significantly upregulated mRNAs and one overexpressed miRNA in the **SOY-F** group after canonical DE analyses. Such difference in the observed post-transcriptional regulation and differential expression of genes between male and female piglets could be associated to sex-specific metabolic responses to n-6/n-3 PUFA ratio. Multiple reports have demonstrated sex-dependent gene regulation guided by miRNAs, transcription factors and other small non-coding RNAs activity, which might underline sex-specific regulatory processes in both health and disease states[33,34]. In addition, there is evidence showing the relationship between sex, sex hormones, and

regulation of biological processes, including lipid-related and inflammatory-induced immunity and health disorders in humans[35–37]. Likewise, age could also be a determinant factor to the expression of protein-coding genes and miRNAs[38,39]. Regarding the PUFAs considered in our experiment, the two omega PUFA series (n-3 and n-6) have antagonistic inflammatory effect and may produce metabolites that can regulate and balance one another[40]. Additional intrinsic factors such as genomics differences, as well as nutritional interventions have the possibility to change, rewire, and/or influence the overall expression profile of mRNAs and miRNAs[41]. Regardless, EISA was able to provide an additional perspective on the miRNA-driven regulation of gene expression that is complimentary to standard differential expression analyses.

4.2. Target prediction highlighted regulatory function of miRNAs in response to PUFAs

In **SOY-M** piglets, most of the genes displaying the top 5% most negative PTc scores with at least 1.5-fold exonic fraction reduction (8 out of 11, 77.73%) showed at least one binding site for the majority of significantly upregulated miRNAs (24 out of 33, 72.73%). The *ssc-miR-29b* was predicted to bind to 6 out of the 8 targeted mRNA genes and could potentially regulate their expression (**Additional Table 5b**). The *miR-29b*, a member of the *miR-29* family, targets several mRNAs modulating a wide range of physiological processes including cell proliferation and apoptosis[42] or angiogenesis[43]. Overexpression of *miR-29b* could be related to the excess of n-6-derived metabolites in piglets fed with SOY diet, which might stimulate angiogenesis by promoting oncogenic growth factors expression[44]. Previous reports have also revealed that *miR-29b* can regulate the expression of pro-inflammatory cytokines and its relationship with inflammation and the immune response[45].

Besides, 60 out of 97 (61.86%) genes highlighted by EISA as post-transcriptionally downregulated in **LIN-M** piglets were predicted as targets of at least one among all the upregulated miRNAs in **LIN-M** piglets, and 18 of these were linked to *ssc-miR-532-3p*

(**Additional Table 6b**). Several studies have shown the anti-cancer properties of *miR-532-3p*, which can inhibit and suppress the malignancy, metastasis and proliferation of different cancer cell types (i.e., ovary, prostate, skin, lymph nodes)[46–48]. These anti-carcinogenic characteristics could be attributed and might be triggered by the antioxidant and anti-inflammatory functions of n-3 PUFAs[49]. Our results also highlighted 6 genes that could be targeted by the upregulated miRNA *ssc-miR-142-5p* in **SOY-F** piglets. This miRNA has been related to human chronic inflammatory and autoimmune diseases and can be induced by immune response regulators such as interleukin-4 and interleukin-13[50,51]. Although the detected miRNA was found to be upregulated in **SOY-F**, its expression was generally low (**Additional Table 10**). While the detected post-transcriptional signals might be weak, this miRNA still was predicted to target a few mRNAs that belonged to the strongest observed downregulation based on ΔEx values and PTc scores in **SOY-F** piglets (**Additional Table 11**), while such effect was not captured by canonical differential expression analyses. This demonstrates the importance of performing analyses to gene expression data other than DE analyses, in order to provide a more comprehensive landscape of the regulatory effects at play.

It is worth noting that, for some of the predicted miRNA-mRNA interactions, there was a clear negative correlation between the given miRNA and the targeted mRNA at the exonic level, as expected for any post-transcriptional regulatory effect happening in the cytoplasm after intron splicing. In this way, when analyzing the correlation between the putative regulatory miRNA and the targeted mRNA at the intronic level, the observed anticorrelation was lost or even of opposite sign compared to that of the exonic fraction. This is the case, for instance, of *ENHO*, *HOXD8*, *EPHB2* and *ST8SIA2* in **SOY-M** piglets (**Additional Table 7a**), or *CHP2* and *CTTNBP2NL* in **LIN-M** piglets (**Additional Table 7b**). The observed predominance of gene expression anticorrelation between these mRNAs and some of their targeting miRNAs at the exonic but not at the intronic level might be indeed a good indicator of the reliability of the predicted interaction, as any no intronic response should be expected, in principle, driven by miRNAs acting at the post-transcriptional level.

4.3. Association between genes with the strongest post-transcriptional downregulation and biological processes related to lipid metabolism and immunomodulation

EISA highlighted multiple genes with relevant post-transcriptional downregulation in **LIN-M** vs **SOY-M**. The *ST8SIA2* gene displayed one of the most negative PTc scores. Additionally, it was detected as significantly downregulated by canonical DE analyses, and harbored the highest number of putative binding sites for thirteen out of the 33 upregulated miRNAs in **SOY-M** piglets. The *ST8SIA2* gene encodes a type II membrane protein that catalyzes the transfer of sialic acid to N-linked oligosaccharides and glycoproteins[52]. This function is relevant since studies have shown that a deficiency in sialic acid or N-glycans is associated with oxidative[53] and inflammatory stress[54], which could be expected in soy-based diets where the n-6/n-3 PUFA ratio is increased. Reports on mice have shown that deficiency of *ST8SIA2*, combined with the decreased expression of insulin growth-like factor 1 receptor, is correlated to lethality[55]. This gene was also found to be targeted by the single one overexpressed miRNA in **SOY-F** piglets (*ssc-miR-142-5p*, **Additional Table 10**).

Other relevant genes that were both post-transcriptionally repressed and significantly downregulated after EISA and canonical DE analyses were the energy homeostasis associated (*ENHO*) and potassium channel tetramerization domain containing 4 (*KCTD4*) genes. *ENHO* encodes a peptide hormone called adropin, which plays a role in regulating lipid and glucose homeostasis and prevents hyperinsulinemia, dyslipidemia, and impaired glucose tolerance[56,57]. Adropin deficiency was linked with increased adiposity, insulin resistance, and metabolic defects[58,59]. Besides, *KCTD4* belongs to a gene family that is associated to cancer, neurological diseases, obesity and metabolic disorders[60–62].

We also detected several other miRNA-targeted transcripts with negative PTc scores correlated to specific lipid-related biological processes such as ephrin type-B receptor 2 (*EPBH2*) which regulates key proteins involved in maintaining lipid and glycogen homeostasis[63,64] or *HOXD8*, involved in carcinogenesis, tumor suppression[65] and

adipogenesis regulation[66]. Nevertheless, the EISA approach also highlighted lipid-related genes such as *COL22A1* and *ALB* as post-transcriptionally downregulated but that did not show a characteristic anticorrelation with upregulated miRNAs putatively targeting them, as shown in **Figure 2c**. Even though they harbored miRNA binding sites, their expression showed confounding patterns that might have elicited a false positive detection by EISA and/or obtained a false interaction by miRNA binding site prediction[67].

With regard to the opposite **SOY-M** vs **LIN-M** contrast, the mRNA with the most negative PTc score and that gathered putative binding sites to fifteen upregulated miRNAs was *TMEM71* (**Additional Table 6a**). This gene encodes a transmembrane protein that is involved in immune and inflammatory response[68]. *CHP2*, which is associated with cancer progression and cellular functions regulation [69,70] and could be inhibited by PUFAs[71], further displayed a clear post-transcriptional downregulation, demonstrating a strong negative correlation with *ssc-miR-532-5p* at the exonic level (**Additional Table 7b**). Besides, arrestin domain containing 2 (*ARRDC2*) plays a vital role in G-protein-coupled receptor signaling and cancer progression. The malfunction of this α-arrestin could lead to diabetes, obesity, cardiovascular diseases[72]. Furthermore, our findings included multiple cluster of differentiation (*CD*) genes, including *CD3*, *CD7*, *CD22*, *CD48*, *CD68*, *CD86*, and *CD163*, which are commonly associated with cytokine generation, T-cell regulation, cancer immunotherapy, immunological response, and/or inflammation[73,74]. Nevertheless, EISA provided a few more genes that were associated to immune system function such as: interleukin-2 receptor α (*IL2RA*)[75]; interferon regulatory factor 4 (*IRF4*)[76]; kinesin family member 21B (*KIF21B*)[77], G protein-coupled receptor 176 (*GPR176*)[78]; and heme oxygenase 1 (*HMOX1*)[79].

Based on our findings, we also obtained mRNAs that were associated with, but not limited to, lipid metabolism, lipoprotein metabolism, atherosclerosis and obesity, such as: porcine-specific cytochrome P450 family 3 subfamily A member 22 (*CYP3A22*)[80]; cadherin related family member 1 (*CDHR1*) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (*PIK3CD*)[81,82]. We also obtained a few genes with negative PTc scores that were not clearly downregulated by upregulated miRNAs – *LIPG* and *ADIPOQ* genes. Likewise, EISA provided some mRNA genes that were post-

transcriptionally repressed and significantly downregulated after DE analysis, but were not targeted by any upregulated miRNAs. Although miRNAs are important post-transcriptional regulators, other post-transcriptional effectors such as long non-coding RNAs, circular RNAs, or RNA binding proteins may also be at play[83,84].

4.4. PUFA abundance in skeletal muscle is associated with the expression of downregulated mRNAs and upregulated miRNAs

Correlation analyses allowed us to link the expression profile of genes showing high post-transcriptional signals, upregulated miRNAs, and the analyzed PUFAs profiles of porcine skeletal muscle. In general, the selected mRNAs and miRNAs showed a negative relationship in both **SOY-M** and **LIN-M** piglets (**Fig. 2c** and **Additional Figure 1c**), as the overexpression of these miRNAs could potentially suppress or downregulate the targeted mRNA transcripts[1]. We observed a clear cluster of positive correlation (**SOY-M** piglets) among mRNAs with strong post-transcriptional downregulation and n-3 PUFAs such as ALA, ETA, and EPA. Conversely, n-6 PUFAs showed the opposite, mostly driven by LA and GLA fatty acids. The observed association between PUFAs concentration and expression of mRNAs and miRNAs agrees well with previous reports on the influence of PUFAs in altering gene regulatory networks[85,86].

Using the same piglet population as in the current study, our previous report[17] showed how the relative proportions of n-3 PUFAs (i.e., ALA, ETA, EPA and DHA) were significantly higher in LD skeletal muscle in LIN compared to SOY diets in piglets, and no significant differences were observed among the n-6 PUFAs. This might explain the weaker correlation driven by n-6 PUFAs on mRNAs and miRNAs obtained in the present study. Moreover, as previously reported[17], the dietary oils that were incorporated in sow's gestation and lactation diets were soybean oil and linseed oil. Fatty acid composition analyses also showed that soybean oil contains high proportion of the LA n-6 PUFA, while linseed oil is composed of mostly the n-3 PUFA ALA [17]. Our results highlighted ALA (n-3) and LA (n-6) as two of the main drivers of the overall phenotypic variance observed in

porcine skeletal muscle in response to changes in PUFA composition in piglet's diet. As the most abundant n-3 and n-6 PUFAs in linseed (ALA) and soybean (LA) oils, they can modulate gene expression related to their antagonistic inflammatory functions and lipid-related metabolic processes[40,87]. Studies on mice demonstrated how varying concentrations of ALA and LA might differentially influence the expression profiles of genes and proteins in muscle tissue[88]. Besides, other additional relevant PUFAs we found were ETA (n-3) which is able trigger the downregulation of mRNAs and transcription factors related to inflammation[89,90], EPA (n-3) which modulates peroxisome proliferator-activated receptors and genes related to dyslipidemia and inflammation[91], or GLA (n-6), that is able to decrease the expression of pro-inflammatory cytokines and related mediators [92].

5. Conclusion

The use of EISA highlighted post-transcriptional changes in mRNA genes expressed in porcine skeletal muscle. Although there was a limited overlap between EISA and canonical DE analyses outputs, EISA provided additional and biologically meaningful information about changes in mRNA expression that were not captured initially, coupled with miRNA-driven repression evidence. Individual n-3 and n-6 PUFAs may play a vital role in gene and miRNA expression and regulation. Both EISA and differential expression analyses highlighted genes and miRNAs that were associated to lipid-mediated and inflammatory-induced biological processes, which could be attributed to the pro- and anti-inflammatory functions of n-6 and n-3 PUFAs, respectively.

Supplementary Materials

Additional Figure 1a. Heatmap plot showing correlation values among mRNA genes with the top 5% negative PTc scores and at least 1.5-fold reduction in ΔEx values and PUFA profiles of *longissimus dorsi* skeletal muscle in **SOY-M** vs **LIN-M** piglets.

Additional Figure 1b. Heatmap plot showing correlation values among upregulated miRNAs and PUFA profiles of *longissimus dorsi* skeletal muscle in **SOY-M** vs **LIN-M** piglets.

Additional Figure 1c. Heatmap plot showing correlation values among mRNA genes with the top 5% negative PTc scores and at least 1.5-fold reduction in ΔEx values and upregulated miRNAs in **SOY-M** vs **LIN-M** piglets.

Additional Figure 2a. Heatmap plot showing correlation values among mRNA genes with the top 5% negative PTc scores and at least 1.5-fold reduction in ΔEx values and PUFA profiles of *longissimus dorsi* skeletal muscle in **SOY-F** vs **LIN-F** piglets.

Additional Figure 2b. Heatmap plot showing correlation values among upregulated miRNAs and PUFA profiles of *longissimus dorsi* skeletal muscle in **SOY-F** vs **LIN-F** piglets.

Additional Figure 2c. Heatmap plot showing correlation values among mRNA genes with the top 5% negative PTc scores and at least 1.5-fold reduction in ΔEx values and upregulated miRNAs in **SOY-F** vs **LIN-F** piglets.

Additional Table 1a. Measured* relative proportion (%) of polyunsaturated fatty acids in *longissimus dorsi* skeletal muscle of **SOY** and **LIN** piglets.

Additional Table 1b. Meta-data including treatment allotment, sex classification, and batch grouping recorded in piglets*.

Additional Table 2a. Differential expression analysis (q-value < 0.05) of RNA-Seq data using edgeR tool in **SOY-M** piglets with respect to **LIN-M** piglets. In bold are differentially expressed genes (N=221) with |FC| > 1.5 and q-value < 0.05.

Additional Table 2b. Differential expression analysis of RNA-Seq data using *edgeR* tool in **SOY-M** piglets with respect to **LIN-M** piglets. DE results showing the whole list of expressed results showing the whole list of expressed genes with an average expression above 1 CPM in at least 50% of samples within each group (N = 11,055).

Additional Table 3. Differential expression analysis of microRNAs using the *edgeR* tool in **SOY-M** piglets with respect to **LIN-M** piglets. In bold are differentially expressed microRNAs (N=62) with |FC| > 1.5 and *q*-value <0.05.

Additional Table 4. Genes downregulated in **SOY-M piglets** with respect to **LIN-M** piglets and with the top 5% most negative post-transcriptional (PTc) scores and at least 1.5-fold exonic fraction (ΔEx) reduction from *longissimus dorsi* skeletal muscle samples.

Additional Table 5a. Binding sites in the 3'-UTRs of mRNA genes (with the top 5% negative PTc scores and at least 2-fold reduction in the exonic fraction) predicted as targets (N = 8) of significantly upregulated miRNAs (N = 24) expressed in the *longissimus dorsi* skeletal muscle samples in **SOY-M** piglets, with respect to **LIN-M** piglets.

Additional Table 5b. Summary of targeted mRNA genes by each upregulated miRNAs in **SOY-M** piglets with respect to **LIN-M** piglets.

Additional Table 5c. Summary of the associated miRNAs and the binding sites in the 3' UTRs of targeted mRNA genes in **SOY-M** piglets with respect to **LIN-M** piglets.

Additional Table 6b. Summary of targeted mRNA genes per upregulated miRNAs in **LIN-M** piglets with respect to **SOY-M** piglets.

Additional Table 6c. Summary of the associated miRNAs and the binding sites in the 3' UTRs of targeted mRNA genes in **LIN-M** piglets with respect to **SOY-M** piglets.

Additional Table 7a. Exonic and intronic correlation values and correlation change of targeted mRNA genes and upregulated miRNAs in **SOY-M** piglets with respect to for **LIN-M** piglets.

Additional Table 7b. Exonic and intronic correlation values and correlation change of targeted mRNA genes and upregulated miRNAs in **LIN-M** piglets with respect to for **SOY-M** piglets.

Additional Table 8. List of Gene Ontology (GO) terms related to DEGs in **SOY-M** and **LIN-M** piglets.

Additional Table 9a. Differential expression analysis of RNA-Seq data using edgeR tool in **SOY-F** piglets with respect to **LIN-F** piglets. In bold are differentially expressed genes (N=33) with |FC| > 1.5 and *q*-value < 0.05.

Additional Table 9b. Differential expression analysis of RNA-Seq data using edgeR tool in **SOY-F** piglets, with respect to **LIN-F** piglets. DE results showing the whole list of expressed results showing the whole list of expressed genes with an average expression above 1 CPM in at least 50% of samples within each group (N = 11,025).

Additional Table 10. Differential expression analysis of microRNAs using the edgeR tool in **SOY-F** piglets, with respect to **LIN-F** piglets. In bold, differentially expressed microRNAs (N=1) with |FC| > 1.5 and q-value <0.05.

Additional Table 11. Genes downregulated in **SOY-F piglets** with respect to **LIN-F** piglets and with the top 5% most negative post-transcriptional (PTc) scores and at least 1.5-fold exonic fraction (ΔEx) reduction from *longissimus dorsi* skeletal muscle samples.

Additional Table 12a. Binding sites in the 3'-UTRs of mRNA genes (with the top 5% negative PTc scores and at least 1.5-fold reduction in the exonic fraction) predicted as targets (N = 6) of significantly upregulated miRNAs (N = 1) expressed in the *longissimus dorsi* skeletal muscle samples in **SOY-F** piglets, with respect to **LIN-F** piglets.

Additional Table 12b. Summary of the *ssc-miR-142-5p* miRNA and the binding sites in the 3'-UTRs of targeted mRNA genes in **SOY-F** piglets, with respect to **LIN-F** piglets.

Additional Table 13. Exonic, intronic, and change in correlation values of targeted genes and upregulated miRNAs in **SOY-F** piglets, with respect to for **LIN-F** piglets.

Additional Table 14. List of Gene Ontology (GO) terms related to DEGs in **SOY-F** and **LIN-F** piglets.

Data availability: TBA

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References

(1) Bartel, D. P. Metazoan MicroRNAs. *Cell* **2018**, *173* (1), 20–51. https://doi.org/10.1016/j.cell.2018.03.006.

(2) Filipowicz, W.; Bhattacharyya, S. N.; Sonenberg, N. Mechanisms of Post-Transcriptional Regulation by MicroRNAs: Are the Answers in Sight? *Nat. Rev. Genet.* **2008**, *9* (2), 102–114. https://doi.org/10.1038/nrg2290.

(3) Naeli, P.; Winter, T.; Hackett, A. P.; Alboushi, L.; Jafarnejad, S. M. The Intricate Balance between MicroRNA-induced MRNA Decay and Translational Repression. *FEBS J.* **2022**. https://doi.org/10.1111/febs.16422.

(4) Horodyska, J.; Wimmers, K.; Reyer, H.; Trakooljul, N.; Mullen, A. M.; Lawlor, P. G.; Hamill, R. M. RNA-Seq of Muscle from Pigs Divergent in Feed Efficiency and Product Quality Identifies Differences in Immune Response, Growth, and Macronutrient and Connective Tissue Metabolism. *BMC Genomics* **2018**, *19* (1), 791. https://doi.org/10.1186/s12864-018-5175-y.

(5) Xing, K.; Liu, H.; Zhang, F.; Liu, Y.; Shi, Y.; Ding, X.; Wang, C. Identification of Key Genes Affecting Porcine Fat Deposition Based on Co-Expression Network Analysis of Weighted Genes. *J. Anim. Sci. Biotechnol.* **2021**, *12* (1), 100. https://doi.org/10.1186/s40104-021-00616-9.

(6) Ramayo-Caldas, Y.; Mach, N.; Esteve-Codina, A.; Corominas, J.; Castelló, A.; Ballester, M.; Estellé, J.; Ibáñez-Escriche, N.; Fernández, A. I.; Pérez-Enciso, M.; Folch, J. M. Liver Transcriptome Profile in Pigs with Extreme Phenotypes of Intramuscular Fatty Acid Composition. *BMC Genomics* **2012**, *13* (1), 547. https://doi.org/10.1186/1471-2164-13-547.

(7) McDermaid, A.; Monier, B.; Zhao, J.; Liu, B.; Ma, Q. Interpretation of Differential Gene Expression Results of RNA-Seq Data: Review and Integration. *Brief. Bioinform.* **2019**, *20* (6), 2044–2054. https://doi.org/10.1093/bib/bby067.

(8) Costa-Silva, J.; Domingues, D.; Lopes, F. M. RNA-Seq Differential Expression Analysis: An Extended Review and a Software Tool. *PLoS One* **2017**, *12* (12), e0190152. https://doi.org/10.1371/journal.pone.0190152.

(9) Frazee, A. C.; Sabunciyan, S.; Hansen, K. D.; Irizarry, R. A.; Leek, J. T. Differential Expression Analysis of RNA-Seq Data at Single-Base Resolution. *Biostatistics* **2014**, *15* (3), 413–426. https://doi.org/10.1093/biostatistics/kxt053.

(10) Mármol-Sánchez, E.; Cirera, S.; Zingaretti, L. M.; Jacobsen, M. J.; Ramayo-Caldas, Y.; Jørgensen, C. B.; Fredholm, M.; Cardoso, T. F.; Quintanilla, R.; Amills, M. Modeling MicroRNA-driven Post-transcriptional Regulation Using Exon–Intron Split Analysis in Pigs. *Anim. Genet.* **2022**, *00*, 1–14. https://doi.org/10.1111/age.13238.

(11) Gaidatzis, D.; Burger, L.; Florescu, M.; Stadler, M. B. Analysis of Intronic and Exonic Reads in RNA-Seq Data Characterizes Transcriptional and Post-Transcriptional Regulation. *Nat. Biotechnol.* **2015**, *33* (7), 722–729. https://doi.org/10.1038/nbt.3269.

(12) Ntambi, J. M.; Bené, H. Polyunsaturated Fatty Acid Regulation of Gene Expression. *J. Mol. Neurosci.* **2001**, *16* (2–3), 273–278. https://doi.org/10.1385/JMN:16:2-3:273.

(13) Georgiadi, A.; Kersten, S. Mechanisms of Gene Regulation by Fatty Acids. *Adv. Nutr.* **2012**, *3* (2), 127–134. https://doi.org/10.3945/an.111.001602.

(14) Sakayori, N.; Katakura, M.; Hamazaki, K.; Higuchi, O.; Fujii, K.; Fukabori, R.; Iguchi, Y.; Setogawa, S.; Takao, K.; Miyazawa, T.; Arita, M.; Kobayashi, K. Maternal Dietary Imbalance between Omega-6 and Omega-3 Fatty Acids Triggers the Offspring's Overeating in Mice. *Commun. Biol.* **2020**, *3* (1), 473. https://doi.org/10.1038/s42003-020-01209-4.

(15) Zivkovic, A. M.; Telis, N.; German, J. B.; Hammock, B. D. Dietary Omega-3 Fatty Acids Aid in the Modulation of Inflammation and Metabolic Health. *Calif. Agric.* **2011**, *65* (3), 106–111. https://doi.org/10.3733/ca.v065n03p106.

(16) Nguyen, T. X.; Agazzi, A.; Comi, M.; Bontempo, V.; Guido, I.; Panseri, S.; Sauerwein, H.; Eckersall, P. D.; Burchmore, R.; Savoini, G. Effects of Low Ω6:Ω3 Ratio in Sow Diet and Seaweed Supplement in Piglet Diet on Performance, Colostrum and Milk Fatty Acid Profiles, and Oxidative Status. *Animals* **2020**, *10* (11), 2049. https://doi.org/10.3390/ani10112049.

(17) Manaig, Y.; Sandrini, S.; Panseri, S.; Tedeschi, G.; Folch, J.; Sánchez, A.; Savoini, G.; Agazzi, A. Low N-6/n-3 Gestation and Lactation Diets Influence Early Performance, Muscle and Adipose Polyunsaturated Fatty Acid Content and Deposition, and Relative Abundance of Proteins in Suckling Piglets. *Molecules* **2022**, *27* (9), 2925. https://doi.org/10.3390/molecules27092925.

(18) Andrews, S. FastQC: A Quality Control Tool for High Throughput Sequence Data http://www.bioinformatics.babraham.ac.uk./projects/fastqc/.

(19) Martin, M. Cutadapt Removes Adapter Sequences from High-Throughput Sequencing Reads. *EMBnet.journal* **2011**, *17* (1), 10. https://doi.org/10.14806/ej.17.1.200.

(20) Kim, D.; Paggi, J. M.; Park, C.; Bennett, C.; Salzberg, S. L. Graph-Based Genome Alignment and Genotyping with HISAT2 and HISAT-Genotype. *Nat. Biotechnol.* **2019**, *37* (8), 907–915. https://doi.org/10.1038/s41587-019-0201-4.

(21) Langmead, B.; Trapnell, C.; Pop, M.; Salzberg, S. L. Ultrafast and Memory-Efficient Alignment of Short DNA Sequences to the Human Genome. *Genome Biol.* **2009**, *10* (3), R25. https://doi.org/10.1186/gb-2009-10-3-r25.

(22) Mármol-Sánchez, E.; Ramayo-Caldas, Y.; Quintanilla, R.; Cardoso, T. F.; González-Prendes, R.; Tibau, J.; Amills, M. Co-Expression Network Analysis Predicts a Key Role of MicroRNAs in the Adaptation of the Porcine Skeletal Muscle to Nutrient Supply. *J. Anim. Sci. Biotechnol.* **2020**, *11* (1), 10.

https://doi.org/10.1186/s40104-019-0412-z.

(23) Warr, A.; Affara, N.; Aken, B.; Beiki, H.; Bickhart, D. M.; Billis, K.; Chow, W.; Eory, L.; Finlayson, H. A.; Flicek, P.; Girón, C. G.; Griffin, D. K.; Hall, R.; Hannum, G.; Hourlier, T.; Howe, K.; Hume, D. A.; Izuogu, O.; Kim, K.; Koren, S.; Liu, H.; Manchanda, N.; Martin, F. J.; Nonneman, D. J.; O'Connor, R. E.; Phillippy, A. M.; Rohrer, G. A.; Rosen, B. D.; Rund, L. A.; Sargent, C. A.; Schook, L. B.; Schroeder, S. G.; Schwartz, A. S.; Skinner, B. M.; Talbot, R.; Tseng, E.; Tuggle, C. K.; Watson, M.; Smith, T. P. L.; Archibald, A. L. An Improved Pig Reference Genome Sequence to Enable Pig Genetics and Genomics Research. *Gigascience* **2020**, *9* (6). https://doi.org/10.1093/gigascience/giaa051.

(24) Liao, Y.; Smyth, G. K.; Shi, W. FeatureCounts: An Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features. *Bioinformatics* **2014**, *30* (7), 923–930. https://doi.org/10.1093/bioinformatics/btt656.

(25) Robinson, M. D.; Oshlack, A. A Scaling Normalization Method for Differential Expression Analysis of RNA-Seq Data. *Genome Biol.* **2010**, *11* (3), R25. https://doi.org/10.1186/gb-2010-11-3-r25.

(26) Robinson, M. D.; McCarthy, D. J.; Smyth, G. K. EdgeR: A Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data. *Bioinformatics* **2010**, *26* (1), 139–140. https://doi.org/10.1093/bioinformatics/btp616.

(27) Benjamini, Y.; Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B* **1995**, *57* (1), 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

(28) Pillman, K. A.; Scheer, K. G.; Hackett-Jones, E.; Saunders, K.; Bert, A. G.; Toubia, J.; Whitfield, H. J.; Sapkota, S.; Sourdin, L.; Pham, H.; Le, T. D.; Cursons, J.; Davis, M. J.; Gregory, P. A.; Goodall, G. J.; Bracken, C. P. Extensive Transcriptional Responses Are Co-Ordinated by MicroRNAs as Revealed by Exon–Intron Split Analysis (EISA). *Nucleic Acids Res.* **2019**, *47* (16), 8606–8619.

https://doi.org/10.1093/nar/gkz664.

(29) Marco, A. SeedVicious: Analysis of MicroRNA Target and near-Target Sites. *PLoS One* **2018**, *13* (4), e0195532. https://doi.org/10.1371/journal.pone.0195532.

(30) Kozomara, A.; Birgaoanu, M.; Griffiths-Jones, S. MiRBase: From MicroRNA Sequences to Function. *Nucleic Acids Res.* **2019**, *47* (D1), D155–D162. https://doi.org/10.1093/nar/gky1141.

(31) Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N. S.; Wang, J. T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* **2003**, *13* (11), 2498–2504. https://doi.org/10.1101/gr.1239303.

(32) Bindea, G.; Mlecnik, B.; Hackl, H.; Charoentong, P.; Tosolini, M.; Kirilovsky, A.; Fridman, W.-H.; Pagès, F.; Trajanoski, Z.; Galon, J. ClueGO: A Cytoscape Plug-in to Decipher Functionally Grouped Gene Ontology and Pathway Annotation Networks. *Bioinformatics* **2009**, *25* (8), 1091–1093. https://doi.org/10.1093/bioinformatics/btp101.

(33) Gershoni, M.; Pietrokovski, S. The Landscape of Sex-Differential Transcriptome and Its Consequent Selection in Human Adults. *BMC Biol.* **2017**, *15* (1), 7. https://doi.org/10.1186/s12915-017-0352-z.

(34) Rounge, T. B.; Umu, S. U.; Keller, A.; Meese, E.; Ursin, G.; Tretli, S.; Lyle, R.; Langseth, H. Circulating Small Non-Coding RNAs Associated with Age, Sex, Smoking, Body Mass and Physical Activity. *Sci. Rep.* **2018**, *8* (1), 17650. https://doi.org/10.1038/s41598-018-35974-4.

(35) Hägg, S.; Jylhävä, J. Sex Differences in Biological Aging with a Focus on Human Studies. *Elife* **2021**, *10*. https://doi.org/10.7554/eLife.63425.

(36) Phelps, T.; Snyder, E.; Rodriguez, E.; Child, H.; Harvey, P. The Influence of Biological Sex and Sex Hormones on Bile Acid Synthesis and Cholesterol Homeostasis. *Biol. Sex Differ.* **2019**, *10* (1), 52. https://doi.org/10.1186/s13293-

019-0265-3.

(37) Klein, S. L.; Flanagan, K. L. Sex Differences in Immune Responses. *Nat. Rev. Immunol.* **2016**, *16* (10), 626–638. https://doi.org/10.1038/nri.2016.90.

(38) Huan, T.; Chen, G.; Liu, C.; Bhattacharya, A.; Rong, J.; Chen, B. H.; Seshadri, S.; Tanriverdi, K.; Freedman, J. E.; Larson, M. G.; Murabito, J. M.; Levy, D. Age-Associated MicroRNA Expression in Human Peripheral Blood Is Associated with All-Cause Mortality and Age-Related Traits. *Aging Cell* **2018**, *17* (1), e12687. https://doi.org/10.1111/acel.12687.

(39) Meder, B.; Backes, C.; Haas, J.; Leidinger, P.; Stähler, C.; Großmann, T.; Vogel, B.; Frese, K.; Giannitsis, E.; Katus, H. A.; Meese, E.; Keller, A. Influence of the Confounding Factors Age and Sex on MicroRNA Profiles from Peripheral Blood. *Clin. Chem.* **2014**, *60* (9), 1200–1208. https://doi.org/10.1373/clinchem.2014.224238.

(40) Schmitz, G.; Ecker, J. The Opposing Effects of N-3 and n-6 Fatty Acids. *Prog. Lipid Res.* **2008**, *47* (2), 147–155. https://doi.org/10.1016/j.plipres.2007.12.004.

(41) Butler, G. Manipulating Dietary PUFA in Animal Feed: Implications for Human Health. *Proc. Nutr. Soc.* **2014**, *73* (1), 87–95. https://doi.org/10.1017/S0029665113003790.

(42) Chung, H. K.; Rao, J. N.; Wang, J.-Y. Regulation of Gut Barrier Function by RNA-Binding Proteins and Noncoding RNAs. In *Comprehensive Pharmacology*; Elsevier, 2022; pp 194–213. https://doi.org/10.1016/B978-0-12-820472-6.00059-1.

(43) Hasegawa, T.; Lewis, H.; Esquela-Kerscher, A. The Role of Noncoding RNAs in Prostate Cancer. In *Translating MicroRNAs to the Clinic*; Laurence, J., Ed.; Academic Press, 2017; pp 329–369. https://doi.org/10.1016/B978-0-12-800553-8.00012-3.

(44) Kang, J. X.; Liu, A. The Role of the Tissue Omega-6/Omega-3 Fatty Acid Ratio in Regulating Tumor Angiogenesis. *Cancer Metastasis Rev.* **2013**, *32* (1–2), 201–

210. https://doi.org/10.1007/s10555-012-9401-9.

(45) Zhang, Z.; Yao, Z.; Zhang, R. Noncoding RNA-Targeted Therapeutics in Autoimmune Diseases: From Bench to Bedside. In *The Epigenetics of Autoimmunity*; Zhang, R., Ed.; Academic Press, 2018; pp 359–386. https://doi.org/10.1016/B978-0-12-809912-4.00017-9.

(46) Tuo, X.; Zhou, Y.; Yang, X.; Ma, S.; Liu, D.; Zhang, X.; Hou, H.; Wang, R.; Li, X.; Zhao, L. MiR-532–3p Suppresses Proliferation and Invasion of Ovarian Cancer Cells via GPNMB/HIF-1α/HK2 Axis. *Pathol. - Res. Pract.* **2022**, *237*, 154032. https://doi.org/10.1016/j.prp.2022.154032.

(47) Feng, C.; So, H. Il; Yin, S.; Su, X.; Xu, Q.; Wang, S.; Duan, W.; Zhang, E.; Sun, C.; Xu, Z. MicroRNA-532-3p Suppresses Malignant Behaviors of Tongue Squamous Cell Carcinoma via Regulating CCR7. *Front. Pharmacol.* **2019**, *10*. https://doi.org/10.3389/fphar.2019.00940.

(48) Wa, Q.; Zou, C.; Lin, Z.; Huang, S.; Peng, X.; Yang, C.; Ren, D.; Xu, D.; Guo, Y.; Liao, Z.; Wang, B.; Hu, H.; Huang, S.; He, P. Ectopic Expression of MiR-532-3p Suppresses Bone Metastasis of Prostate Cancer Cells via Inactivating NF-KB Signaling. *Mol. Ther. - Oncolytics* **2020**, *17*, 267–277. https://doi.org/10.1016/j.omto.2020.03.024.

(49) Leghi, G. E.; Muhlhausler, B. S. The Effect of N-3 LCPUFA Supplementation on Oxidative Stress and Inflammation in the Placenta and Maternal Plasma during Pregnancy. *Prostaglandins, Leukot. Essent. Fat. Acids* **2016**, *113*, 33–39. https://doi.org/10.1016/j.plefa.2016.08.010.

(50) Su, S.; Zhao, Q.; He, C.; Huang, D.; Liu, J.; Chen, F.; Chen, J.; Liao, J.-Y.; Cui, X.; Zeng, Y.; Yao, H.; Su, F.; Liu, Q.; Jiang, S.; Song, E. MiR-142-5p and MiR-130a-3p Are Regulated by IL-4 and IL-13 and Control Profibrogenic Macrophage Program. *Nat. Commun.* **2015**, *6* (1), 8523. https://doi.org/10.1038/ncomms9523.

(51) Bayomy, N. R.; Abo Alfottoh, W. M.; Ali Eldeep, S. A.; Ibrahim Mabrouk Mersal, A. M. S.; Abd El- Bary, H. M. A.; Abd El Gayed, E. M. Mir-142-5p as an

Indicator of Autoimmune Processes in Childhood Idiopathic Nephrotic Syndrome and as a Part of MicroRNAs Expression Panels for Its Diagnosis and Prediction of Response to Steroid Treatment. *Mol. Immunol.* **2022**, *141*, 21–32. https://doi.org/10.1016/j.molimm.2021.11.004.

(52) Yang, X.; Li, L.; Chai, X.; Liu, J. The Association between ST8SIA2 Gene and Behavioral Phenotypes in Children with Autism Spectrum Disorder. *Front. Behav. Neurosci.* **2022**, *16*. https://doi.org/10.3389/fnbeh.2022.929878.

(53) Cho, A.; Christine, M.; Malicdan, V.; Miyakawa, M.; Nonaka, I.; Nishino, I.; Noguchi, S. Sialic Acid Deficiency Is Associated with Oxidative Stress Leading to Muscle Atrophy and Weakness in GNE Myopathy. *Hum. Mol. Genet.* **2017**, *26* (16), 3081–3093. https://doi.org/10.1093/hmg/ddx192.

(54) Woodward, A. M.; Lehoux, S.; Mantelli, F.; Di Zazzo, A.; Brockhausen, I.; Bonini, S.; Argüeso, P. Inflammatory Stress Causes N-Glycan Processing Deficiency in Ocular Autoimmune Disease. *Am. J. Pathol.* **2019**, *189* (2), 283–294. https://doi.org/10.1016/j.ajpath.2018.10.012.

(55) Ikegami, K.; Saigoh, K.; Fujioka, A.; Nagano, M.; Kitajima, K.; Sato, C.; Masubuchi, S.; Kusunoki, S.; Shigeyoshi, Y. Effect of Expression Alteration in Flanking Genes on Phenotypes of St8sia2-Deficient Mice. *Sci. Rep.* **2019**, *9* (1), 13634. https://doi.org/10.1038/s41598-019-50006-5.

(56) Kumar, K. G.; Trevaskis, J. L.; Lam, D. D.; Sutton, G. M.; Koza, R. A.; Chouljenko, V. N.; Kousoulas, K. G.; Rogers, P. M.; Kesterson, R. A.; Thearle, M.; Ferrante, A. W.; Mynatt, R. L.; Burris, T. P.; Dong, J. Z.; Halem, H. A.; Culler, M. D.; Heisler, L. K.; Stephens, J. M.; Butler, A. A. Identification of Adropin as a Secreted Factor Linking Dietary Macronutrient Intake with Energy Homeostasis and Lipid Metabolism. *Cell Metab.* **2008**, *8* (6), 468–481. https://doi.org/10.1016/j.cmet.2008.10.011.

(57) Es-haghi, A.; Al-Abyadh, T.; Mehrad-Majd, H. The Clinical Value of Serum Adropin Level in Early Detection of Diabetic Nephropathy. *Kidney Blood Press.*

Res. **2021**, *46* (6), 734–740. https://doi.org/10.1159/000519173.

(58) Ganesh-Kumar, K.; Zhang, J.; Gao, S.; Rossi, J.; McGuinness, O. P.; Halem, H. H.; Culler, M. D.; Mynatt, R. L.; Butler, A. A. Adropin Deficiency Is Associated With Increased Adiposity and Insulin Resistance. *Obesity* **2012**, *20* (7), 1394–1402. https://doi.org/10.1038/oby.2012.31.

(59) Chen, S.; Zeng, K.; Liu, Q.; Guo, Z.; Zhang, S.; Chen, X.; Lin, J.; Wen, J.; Zhao, C.; Lin, X.; Gao, F. Adropin Deficiency Worsens HFD-Induced Metabolic Defects. *Cell Death Dis.* **2017**, *8* (8), e3008–e3008. https://doi.org/10.1038/cddis.2017.362.

(60) Liu, Z.; Xiang, Y.; Sun, G. The KCTD Family of Proteins: Structure, Function, Disease Relevance. *Cell Biosci.* **2013**, *3* (1), 45. https://doi.org/10.1186/2045-3701-3-45.

(61) Teng, X.; Aouacheria, A.; Lionnard, L.; Metz, K. A.; Soane, L.; Kamiya, A.; Hardwick, J. M. KCTD : A New Gene Family Involved in Neurodevelopmental and Neuropsychiatric Disorders. *CNS Neurosci. Ther.* **2019**, *25* (7), 887–902. https://doi.org/10.1111/cns.13156.

(62) Ng, M. C. Y.; Tam, C. H. T.; So, W. Y.; Ho, J. S. K.; Chan, A. W.; Lee, H. M.; Wang, Y.; Lam, V. K. L.; Chan, J. C. N.; Ma, R. C. W. Implication of Genetic Variants Near NEGR1 , SEC16B , TMEM18 , ETV5/DGKG , GNPDA2 , LIN7C/BDNF , MTCH2 , BCDIN3D/FAIM2 , SH2B1 , FTO , MC4R , and KCTD15 with Obesity and Type 2 Diabetes in 7705 Chinese. *J. Clin. Endocrinol. Metab.* **2010**, *95* (5), 2418–2425. https://doi.org/10.1210/jc.2009-2077.

(63) Morales, A.; Greenberg, M.; Nardi, F.; Gil, V.; Hayward, S. W.; Crawford, S. E.; Franco, O. E. Loss of Ephrin B2 Receptor (EPHB2) Sets Lipid Rheostat by Regulating Proteins DGAT1 and ATGL Inducing Lipid Droplet Storage in Prostate Cancer Cells. *Lab. Investig.* **2021**, *101* (7), 921–934. https://doi.org/10.1038/s41374-021-00583-9.

(64) Jiang, J.; Wang, Z.-H.; Qu, M.; Gao, D.; Liu, X.-P.; Zhu, L.-Q.; Wang, J.-Z.

Stimulation of EphB2 Attenuates Tau Phosphorylation through PI3K/Akt-Mediated Inactivation of Glycogen Synthase Kinase-3β. *Sci. Rep.* **2015**, *5* (1), 11765. https://doi.org/10.1038/srep11765.

(65) Zhang, Y.; Yu, Y.; Su, X.; Lu, Y. HOXD8 Inhibits the Proliferation and Migration of Triple-Negative Breast Cancer Cells and Induces Apoptosis in Them through Regulation of AKT/MTOR Pathway. *Reprod. Biol.* **2021**, *21* (4), 100544. https://doi.org/10.1016/j.repbio.2021.100544.

(66) Kumar, V.; Sekar, M.; Sarkar, P.; Acharya, K. K.; Thirumurugan, K. Dynamics of HOX Gene Expression and Regulation in Adipocyte Development. *Gene* **2021**, *768*, 145308. https://doi.org/10.1016/j.gene.2020.145308.

(67) Pinzón, N.; Li, B.; Martinez, L.; Sergeeva, A.; Presumey, J.; Apparailly, F.; Seitz, H. MicroRNA Target Prediction Programs Predict Many False Positives. *Genome Res.* **2017**, *27* (2), 234–245. https://doi.org/10.1101/gr.205146.116.

(68) Wang, K.; Huang, R.; Tong, X.; Zhang, K.; Liu, Y.; Zeng, F.; Hu, H.; Jiang, T. Molecular and Clinical Characterization of TMEM71 Expression at the Transcriptional Level in Glioma. *CNS Neurosci. Ther.* **2019**, *25* (9), 965–975. https://doi.org/10.1111/cns.13137.

(69) Di Sole, F.; Vadnagara, K.; Moe, O. W.; Babich, V. Calcineurin Homologous Protein: A Multifunctional Ca 2+ -Binding Protein Family. *Am. J. Physiol. Physiol.* **2012**, *303* (2), F165–F179. https://doi.org/10.1152/ajprenal.00628.2011.

(70) Li, G.-D.; Zhang, X.; Li, R.; Wang, Y.-D.; Wang, Y.-L.; Han, K.-J.; Qian, X.-P.; Yang, C.-G.; Liu, P.; Wei, Q.; Chen, W.-F.; Zhang, J.; Zhang, Y. CHP2 Activates the Calcineurin/Nuclear Factor of Activated T Cells Signaling Pathway and Enhances the Oncogenic Potential of HEK293 Cells. *J. Biol. Chem.* **2008**, *283* (47), 32660–32668. https://doi.org/10.1074/jbc.M806684200.

(71) Tokheim, A. M.; Martin, B. L. Inhibition of Calcineurin by Polyunsaturated Lipids. *Bioorg. Chem.* **2006**, *34* (2), 66–76. https://doi.org/10.1016/j.bioorg.2005.12.002.

(72) Zalewska, M.; Siara, M.; Sajewicz, W. G Protein-Coupled Receptors: Abnormalities in Signal Transmission, Disease States and Pharmacotherapy. *Acta Pol. Pharm. 71* (2), 229–243.

(73) McArdel, S. L.; Terhorst, C.; Sharpe, A. H. Roles of CD48 in Regulating Immunity and Tolerance. *Clin. Immunol.* **2016**, *164*, 10–20. https://doi.org/10.1016/j.clim.2016.01.008.

(74) Zeibig, S.; Büttcher, M.; Goebel, S.; Pauli, J.; Hunger, A.; Ungerer, M.; Gawaz, M.; Münch, G. The Scavenger Receptor CD68 Regulates Platelet Mediated Oxidized Low-Density Lipoprotein (OxLDL) Deposition in Atherosclerotic Vessels at an Early Stage of Atherosclerosis in LDLR-/-/ApoBec-/- Mice. *Cell. Physiol. Biochem.* **2019**, *52* (4), 681–695. https://doi.org/10.33594/000000048.

(75) Buhelt, S.; Laigaard, H.-M.; von Essen, M. R.; Ullum, H.; Oturai, A.; Sellebjerg, F.; Søndergaard, H. B. IL2RA Methylation and Gene Expression in Relation to the Multiple Sclerosis-Associated Gene Variant Rs2104286 and Soluble IL-2Rα in CD8+ T Cells. *Front. Immunol.* **2021**, *12*. https://doi.org/10.3389/fimmu.2021.676141.

(76) Sundararaj, S.; Seneviratne, S.; Williams, S. J.; Enders, A.; Casarotto, M. G. The Molecular Basis for the Development of Adult T-cell Leukemia/Lymphoma in Patients with an <scp> IRF4 ^{K59R} </Scp> Mutation. *Protein Sci.* **2022**, *31* (4), 787–796. https://doi.org/10.1002/pro.4260.

(77) Hooikaas, P. J.; Damstra, H. G.; Gros, O. J.; van Riel, W. E.; Martin, M.; Smits, Y. T.; van Loosdregt, J.; Kapitein, L. C.; Berger, F.; Akhmanova, A. Kinesin-4 KIF21B Limits Microtubule Growth to Allow Rapid Centrosome Polarization in T Cells. *Elife* **2020**, *9*. https://doi.org/10.7554/eLife.62876.

(78) Goto, K.; Doi, M.; Wang, T.; Kunisue, S.; Murai, I.; Okamura, H. G-Protein-Coupled Receptor Signaling through Gpr176, Gz, and RGS16 Tunes Time in the Center of the Circadian Clock [Review]. *Endocr. J.* **2017**, *64* (6), 571–579. https://doi.org/10.1507/endocrj.EJ17-0130.

(79) Gao, M.; Qi, Z.; Deng, M.; Huang, H.; Xu, Z.; Guo, G.; Jing, J.; Huang, X.; Xu, M.; Kloeber, J. A.; Liu, S.; Huang, J.; Lou, Z.; Han, J. The Deubiquitinase USP7 Regulates Oxidative Stress through Stabilization of HO-1. *Oncogene* **2022**, *41* (33), 4018–4027. https://doi.org/10.1038/s41388-022-02403-w.

(80) Konkel, A.; Schunck, W.-H. Role of Cytochrome P450 Enzymes in the Bioactivation of Polyunsaturated Fatty Acids. *Biochim. Biophys. Acta - Proteins Proteomics* **2011**, *1814* (1), 210–222. https://doi.org/10.1016/j.bbapap.2010.09.009.

(81) Ballas, Z. K. The Editors' Choice. *J. Allergy Clin. Immunol.* **2018**, *142* (2), 394–395. https://doi.org/10.1016/j.jaci.2018.06.028.

(82) Virk, R.; Buddenbaum, N.; Al-Shaer, A.; Armstrong, M.; Manke, J.; Reisdorph, N.; Sergin, S.; Fenton, J. I.; Wallace, E. D.; Ehrmann, B. M.; Lovins, H. B.; Gowdy, K. M.; Smith, M. R.; Smith, G. J.; Kelada, S. N. P.; Shaikh, S. R. Obesity Reprograms the Pulmonary Polyunsaturated Fatty Acid-Derived Lipidome, Transcriptome, and Gene-Oxylipin Networks. *J. Lipid Res.* **2022**, *63* (10), 100267. https://doi.org/10.1016/j.jlr.2022.100267.

(83) Bose, R.; Ain, R. Regulation of Transcription by Circular RNAs; 2018; pp 81–94. https://doi.org/10.1007/978-981-13-1426-1_7.

(84) Van Assche, E.; Van Puyvelde, S.; Vanderleyden, J.; Steenackers, H. P. RNA-Binding Proteins Involved in Post-Transcriptional Regulation in Bacteria. *Front. Microbiol.* **2015**, *6*. https://doi.org/10.3389/fmicb.2015.00141.

(85) Xie, Y.; Liu, Z.; Guo, J.; Su, X.; Zhao, C.; Zhang, C.; Qin, Q.; Dai, D.; Zhao, Y.; Wang, Z.; Wang, R.; Zhang, Y.; Su, R.; Wang, Z.; Li, J. MicroRNA-MRNA Regulatory Networking Fine-Tunes Polyunsaturated Fatty Acid Synthesis and Metabolism in the Inner Mongolia Cashmere Goat. *Front. Genet.* **2021**, *12*. https://doi.org/10.3389/fgene.2021.649015.

(86) Zheng, Z.; Ge, Y.; Zhang, J.; Xue, M.; Li, Q.; Lin, D.; Ma, W. PUFA Diets Alter the MicroRNA Expression Profiles in an Inflammation Rat Model. *Mol. Med. Rep.*

2015, *11* (6), 4149–4157. https://doi.org/10.3892/mmr.2015.3318.

(87) Smink, W.; Gerrits, W. J. J.; Gloaguen, M.; Ruiter, A.; Van Baal, J. Linoleic and α-Linolenic Acid as Precursor and Inhibitor for the Synthesis of Long-Chain Polyunsaturated Fatty Acids in Liver and Brain of Growing Pigs. *Animal* **2012**, *6* (2), 262–270. https://doi.org/10.1017/S1751731111001479.

(88) Rajna, A.; Gibling, H.; Sarr, O.; Matravadia, S.; Holloway, G. P.; Mutch, D. M. Alpha-Linolenic Acid and Linoleic Acid Differentially Regulate the Skeletal Muscle Secretome of Obese Zucker Rats. *Physiol. Genomics* **2018**, *50* (8), 580–589. https://doi.org/10.1152/physiolgenomics.00038.2018.

(89) Chen, S.-J.; Chuang, L.-T.; Chen, S.-N. Incorporation of Eicosatrienoic Acid Exerts Mild Anti-Inflammatory Properties in Murine RAW264.7 Cells. *Inflammation* **2015**, *38* (2), 534–545. https://doi.org/10.1007/s10753-014-9960-8.

(90) Jin, X.-J.; Kim, E. J.; Oh, I. K.; Kim, Y. K.; Park, C.-H.; Chung, J. H. Prevention of UV-Induced Skin Damages by 11,14,17-Eicosatrienoic Acid in Hairless Mice In Vivo. *J. Korean Med. Sci.* **2010**, *25* (6), 930. https://doi.org/10.3346/jkms.2010.25.6.930.

(91) Gillies, P. J.; Bhatia, S. K.; Belcher, L. A.; Hannon, D. B.; Thompson, J. T.; Vanden Heuvel, J. P. Regulation of Inflammatory and Lipid Metabolism Genes by Eicosapentaenoic Acid-Rich Oil. *J. Lipid Res.* **2012**, *53* (8), 1679–1689. https://doi.org/10.1194/jlr.M022657.

(92) Youn, K.; Lee, S.; Jun, M. Gamma-Linolenic Acid Ameliorates Aβ-Induced Neuroinflammation through NF-KB and MAPK Signalling Pathways. *J. Funct. Foods* **2018**, *42*, 30–37. https://doi.org/10.1016/j.jff.2017.12.065.

GENERAL DISCUSISION

Chapter 4

Pig or pork in general, ranks among the most significant agricultural livestock animals, including poultry and cattle. It has been considered as one of the main sources of highly concentrated protein for humans. With a total global meat production of 122.5 million tons in 2021, FAO emphasized that the increase in pork production was primarily responsible for the recent rise in global meat production (Food and Agriculture Organization (FAO), 2022). Aside from being the largest exporter of pig meat and pig meat products, the European Union is regarded as the world's second-largest producer of meat behind China (OECD/FAO, 2018).

Meat quality can be influenced and dictated by multiple factors and their relationship, such as breed, genotype, nutrition, slaughtering, and storage conditions. Intramuscular fat and fatty acid composition have been the most two important traits in pork production considering that these two traits can affect the overall juiciness, tenderness, flavor, and sensory quality of pork (Nold, 2006; Rosenvold & Andersen, 2003). Moreover, recent development on porcine breeds have undergone extensive selection in order to boost the production of lean meat and decrease its fat content, which might impact productive and reproductive performance of the animal (Martinsen et al., 2015; Rauw et al., 1998).

Dietary fat intake of pigs directly affects the fatty acid composition of the carcass as it mimics the fatty acid composition of the diet. Polyunsaturated fatty acids (PUFA), particularly the omega-6 (n-6) and omega-3 (n-6) PUFAs and their antagonistic inflammatory functions, have the ability to regulate gene expression and transcription factor activity. This could potentially affect numerous biological processes such as nutrient metabolism, signal-transduction pathways, messenger RNA (mRNA) transcription, microRNA (miRNA) suppression, and protein synthesis (Georgiadi & Kersten, 2012; Ntambi & Bené, 2001).

OMICS technologies are gaining a lot of traction and interest in these recent years in studying the roles and relationships of genome, transcriptomes, proteomes, and metabolomes, that make up the cell, tissue, and organism. It is referred to as a ‘high-dimensional biology’ which is frequently used to increase the understanding of biological

differentiation, phenotypic variation, disease prognosis, and other relevant physiological processes (Horgan & Kenny, 2011; Karahalil, 2016; Romero et al., 2006; Ward, 2014).

In this PhD thesis, we analyzed the effect of dietary lipids, in terms of omega-6/omega-3 polyunsaturated fatty acid ratio, on porcine meat quality relating to intramuscular fat content and composition and the integration of OMICS technologies such as genomics, transcriptomics, and proteomics.

4.1. n-6/n-3 PUFA ratio and porcine productive and reproductive performance

Dietary energy comprises about 75% of the overall cost of swine diets. In diets with low feed intake, particularly in hot weather or in high-producing lactating sows when energy is limited, fats and oils are given to boost energy density since they contain around 2.25 times as much energy as cereal grains (Patience, 2010).

In our study, we looked into the direct effect of high (13:1, **SOY**) and low (4:1, **LIN**) n-6/n-3 PUFA ratios in the sow's diet on the reproductive performance of sows and growth performance of pre-weaning piglets. The dietary treatments had no effect on sow body, weight gain during gestation, or even during lactation period. Even though we did not observe any effect on the total number of piglets born and piglets weaned per sow, the number of dead-born piglets was significantly higher in **SOY** compared to **LIN**. Furthermore, no significant differences in pre-weaning mortality were found between SOY and LIN but the magnitude of difference between the two mortality rates were drastic (22.50% ± 0.06% vs. 11.0% ± 0.06%, respectively). This was in accordance to a recent review of n-3 supplementation on the maternal sow diet and how multiple studies of n-3 PUFA addition have shown beneficial effects on sow reproductive performance in decreasing piglet mortality and further improving the pre- and post-weaning growth rates (Tanghe & De Smet, 2013).

As reported in our current research, the dietary treatments had no significant effect on both litter weight, litter weight gain of the piglets, birth weight, and weaning weight, but a significantly higher individual body weight was detected in **LIN** piglets on d 15 of

lactation. The ADG between **SOY** and **LIN** litters and piglets did not differ from birth to weaning, whereas **LIN** diet increased the ADG of individual piglets within its litter group. These claims were supported by a meta-analysis in human research, which showed that n-3 PUFA supplementation alone enhanced neonatal birth weight and was associated with higher concentrations of the n-3 PUFAs DHA and EPA (Li et al., 2018; Rooke et al., 2001); and in mice wherein the incorporation of n-3 PUFAs also boosted the growth of the fetus and placenta, and it decreased the oxidative breakdown of lipids by boosting the production of antioxidant enzymes in placental zones (Jones et al., 2013). It was also noteworthy that from day 15 till weaning, the weight gain of the piglets in the **SOY** group was higher. Major n-6 PUFAs, such as AA, have a strong correlation with newborn growth, therefore the high AA concentration from the sow diet maybe responsible for this phenomenon. Since n-3 and n-6 PUFAs both act as eicosanoid precursors and have opposing inflammatory roles, synthesis pathways, and oxidation mechanisms, the heterogeneity of the results on piglet growth performance may be attributed to substrate competition that might occur among these two PUFAs (Schmitz & Ecker, 2008).

Research also indicates on how lipids, in general, can improve the average daily gain and further improve the feed conversion rates of the pigs – adding 3% to 5% fat or oil to growing-finishing swine diets will improve feed conversion, stating that for every 1% fat added in growing-finishing pig diets, feed efficiency is improved by 1.8%; and in terms of average daily gain, a percent increase of fat may contribute to an increase of 2% and 1% in growing and late finishing pigs, respectively (De la Llata et al., 2001; DeRouchey et al., 2007; Jung et al., 2003). Addition of fat can also increase the apparent ileal digestibility of amino acids and decrease the feed intake of the pigs (Kil & Stein, 2011).

4.2. n-6/n-3 PUFA ratio and fatty acid profile and meat quality

The term "essential fatty acids" (EFA) refers to those PUFAs that cannot be synthesized by the body yet are essential for health and development. The n-6 and n-3 PUFAs are considered the two families of essential fatty acids (Kaur et al., 2014). As for pigs, like mammals, they lack the desaturase enzyme necessary for the FA *de novo*

synthesis, thus prohibiting them to incorporate double bonds past carbon 9 and 10 of FA. As a result, such requirement of EFA should be obtained from the diet.

In our experiments, we had two pig populations that were used for the analyses. One of which were those animals that were selected from an experimental backcross population of Iberian and Duroc pigs and were given *ad libitum* access to a uniform cereal-based commercial diets (Martínez-Montes et al., 2018). The other set of animals were a group of piglets that were nourished only with sow's colostrum and milk, in which these sows were fed with diets containing n-6/n-3 PUFA ratios of 4:1 (**LIN**) or 13:1 (**SOY**) – these ratios were attained by the addition of either soybean or linseed oil as source of n-6 or n-3 PUFAs, respectively (Manaig et al., 2022).

Although the pigs from the backcross of Iberian and Duroc did not receive any dietary lipid intervention, we were still able to select the animals based on their analyzed fatty acid profile in relation to n-6/n-3 PUFA ratio, filtering the highest and lowest for the trait of interest. Thus, explaining that there might be some cereal or oil ingredients from that commercial diet which could be a source of either n-6 or n-3 PUFA, as it was deposited and detected on the porcine skeletal muscle. Regardless of fat source, *de novo* synthesis of fatty acids is inhibited in favor of direct fatty acid deposition in animal tissue, where 31-40% of dietary fat is transferred to carcass lipids and to the intramuscular fat of muscle tissues, such as the *longissimus dorsi*, semimembranosus, *biceps femoris*, diaphragm, and masseter (Chilliard, 1993; Farnworth & Kramer, 1987; Kloareg et al., 2007; Realini et al., 2010).

On the other hand, the selected piglets have shown a more pronounced differentiation of PUFA content in their muscle and adipose tissues. The fatty acid profiles of the aforementioned tissues displayed a great resemblance to nutritional interventions and the same directions for total n-3, n-6, and n-6/n-3 PUFA ratios. The low n-6/n-3 PUFA ratio in sow diets decreased the n-6/n-3 PUFA content in the muscle and adipose tissues of piglets, which could be mainly driven by the increased proportion of n-3 PUFA in both tissues. According to our findings, piglets from sows fed **SOY** diets had muscle and adipose tissue with n-6/n-3 ratios of 16.45:1 and 14.70:1, respectively, whereas piglets

from sows fed **LIN** diets had an n-6/n-3 ratio of 9.72:1 for muscle tissue and 7.47:1 for adipose tissue. There was also a published study in which they have also used the same ratios that were implemented on our research, in which the low n-6/n-3 ratio has led to the increased level of n-3 PUFAs and decreased n-6/n-3 PUFA ratio in colostrum and milk (Nguyen et al., 2020). Given that the piglets used in this study were only fed sow's milk, these findings further demonstrated how the FA profiles of colostrum and milk were mirrored and were then incorporated into the muscle and subcutaneous fat of suckling piglets. Moreover, the dietary treatments had no effect on the overall level of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), or PUFA in either muscle or adipose tissues, but significant differences were found for the majority of specific n-6 PUFAs and n-3 PUFAs. Additionally, if the nutrient requirement for maintenance is met, the efficiency of dietary fat utilization is at 90%, as most of the fatty acids are minimally hydrogenated or remain unchanged (Mitchaothai et al., 2007; National Research Council, 2012).

Although the effect was not apparent on piglet muscle tissue, the n-6 PUFA concentration in the piglet's adipose tissue decreased after the administration of low n-6/n-3 ratio diet, leading to a lower proportions of linoleic acid (LA), gamma γ-linoleic acid (GLA), and arachidonic acid (AA). Furthermore, the sow diet with low n-6/n-3 ratio contributed toward a universal increase in all the major n-3 PUFAs in the piglets' muscle and adipose tissue. The extent of the variation in the omega ratio content of the diets and how this appeared in the tissue of the piglets were followed by a clearly visible pattern of fatty acid accumulation in the animal, hence, contributing on the utilization of fatty acids in pigs and how they were deposited on the carcass.

While the variations between the fatty acids within adipose tissues in two dietary treatments were more pronounced, the relative proportion of fatty acids identified in muscles was much higher in percentages than in adipose tissue. After birth, the accumulation of body fat occurs through cell hyperplasia (increase in cell number) and hypertrophy (increase in cell size), which leads to the formation of body fat. Adipocyte hyperplasic development is preferred in the early life stages in which these developed lipocytes turn into the primary fat depots that are typically located in perirenal, mesenteric, and intermuscular fat. As the animal grows, they continue to accumulate and manufacture

more fat cells in subcutaneous and intramuscular deposits (Sainz & Hasting, 2000). Therefore, intramuscular fat (IMF) is the last adipocyte depot in animals from a developmental perspective. Typically, fat is deposited in the following order: perirenal, intermuscular, subcutaneous, and then intramuscular. We could speculate that the fat cells in subcutaneous fat have already utilized up all of their storage capacity and have redirected the remaining or excess fat that is still present into the muscle tissue, thus, increasing the amount of fat that is deposited in the *longissimus dorsi* muscle. Since subcutaneous carcass fat and total muscle lipid composition mostly increase with the maturation of pigs, the age (d 26) of the piglets at the time of biological sample collection should also be taken into consideration (Kouba et al., 2003). Additionally, the quantity and metabolic activity of adipocytes, the rate at which muscle tissues grow, the metabolic activity of organs like the liver, and the PUFA concentration of membrane lipids can impact the accumulation of IMF in muscle (Corino et al., 2014; Hocquette et al., 2010).

Sow diet influences the accumulation of fatty acids by piglets through placental lipid transfer. Diets during gestation directly affect sow's milk and piglet's plasma fatty acid composition. This demonstrates yet another way in which maternal adipose tissue serves as the initial reservoir for dietary fatty acids before they are mobilized around farrowing and transmitted to piglets (Amusquivar et al., 2010). According to previous data, the availability and similarity of fatty acids in the human placenta may also be influenced by the composition of the maternal diet (Díaz et al., 2017; Wada et al., 2017).

4.3. n-6/n-3 PUFA ratio and relevant genes and biological processes

The n-6 and n-3 series of PUFA have the ability to regulate gene expression and the activity of transcription factors, potentially affecting the overall nutrient metabolism, regulatory networks, signal-transduction pathways, and mRNA transcription (Butler, 2014; Georgiadi & Kersten, 2012; Ntambi & Bené, 2001). Nowadays, commercial swine feed is mostly composed of cereal and soybean meal, along with plant and plant oil sources which are primarily composed of n-6 PUFA. Consequently, this makes the amount of n-6 in feed mixes about 10 times higher than n-3 PUFA (Gjerlaug-Enger et al., 2015). Moreover, as

the excessive supplementation of n-6 PUFAs becomes more prevalent on pig diets, its continuous ingestion may cause an imbalance between the two PUFAs. Metabolites derived from n-6 and n-3 PUFAs are common precursors of eicosanoids that can regulate inflammation. As such, these two are also antagonistic in their inflammatory nature, with n-6 PUFA acting as pro-inflammatory and n-3 PUFA as anti-inflammatory, respectively, and may potentially induce substrate competition that can potentially affect metabolic health and inflammatory modulation (Sakayori et al., 2020; Zivkovic et al., 2011). Regulation of n-6 PUFA-derived metabolites can be mediated through balancing the ratio between these and n-3 PUFA, which counteract pro-inflammatory responses elicited by the excess of n-6 PUFAs (Dennis & Norris, 2015).

Based on our results from the backcross population of Iberian and Duroc, analyzing the transcriptome of extreme animals for n-6/n-3 PUFA ratio on porcine skeletal muscle allowed us to obtain a total of 432 DEGs, with 157 and 275 genes being upregulated and downregulated in L pigs with respect to H pigs, respectively. Our results also showed GO terms mostly related to muscle growth and differentiation, glucose and lipid metabolism. In this paper, we also listed a few genes whose functions could be influenced and mediated by lipids, related to obesity, regulation of lipogenesis and lipolysis, and cholesterol formation. To highlight a few, our paper reported aryl hydrocarbon receptor nuclear translocator like (*ARNTL*), a gene that regulates the circadian release of PUFAs and modulates feeding behavior in mice, and alongside with forkhead box N2 (*FOXN2*), are associated with obesity (Laufer et al., 2021; Paschos et al., 2012). The diaphanous related formin 1 (*DIAPH1*) gene, which is reported to be involved in lipid metabolism in cattle and is regulated by long chain PUFAs (n-6 and n-3) (Liu et al., 2020). Another gene of interest is the strawberry notch homolog 1 (*SBNO1*) which was was also found to be associated with plasma high-density lipoprotein cholesterol content in GWAS analysis of human metabolic syndrome, whereas the vestigial like family member 2 (*VGLL2*) gene was associated with the fatty acids profile in sheep (Lanktree et al., 2013; Rovadoscki et al., 2018).

Aside from this, the SREBP signaling pathway is another significant GO term that can be highlighted from our findings. The transcription factors known as sterol regulatory-

element binding proteins (SREBPs) control and regulate the expression patterns of genes that are involved in lipid synthesis, energy storage, and cholesterol regulation. When these proteins are activated, they can trigger lipid-mediated cellular stress that can cause metabolic diseases such as obesity, atherosclerosis, diabetes mellitus, inflammation, and organ fibrosis (DeBose-Boyd & Ye, 2018; Shimano & Sato, 2017). Furthermore, our enrichment analysis also highlighted the adenylate cyclase-activating adrenergic receptor signaling pathway. Adrenergic receptors are essential for immunomodulation, modulating stress-induced signals, and behavior modification in response to stress (Gorman & Dunn, 1993; Kolmus et al., 2015). It is also possible that the pro-inflammatory function of n-6 PUFA is related to the stimulation of the SREBP signaling pathway and the adrenergic receptor signaling pathway. In pig adipocyte plasma membrane, the addition of n-6 PUFA enhanced the binding of β-adrenergic receptors and adenylate cyclase activity (Nicolas et al., 1991). In our proteomics study, we concluded that over supplementation of n-6 PUFAs in swine diets can stimulate proteins and protein-coding genes related to innate immune response and acute inflammatory response (Manaig et al., 2022).

Based on *edgeR* analysis, we obtained 221 DEGs, comprising of 174 upregulated and 47 downregulated genes in **SOY-M** pigs with respect to **LIN-M** pigs, and vice versa. Functional enrichment analysis demonstrated a total of 521 significant unique GO terms for the DEGs related to **LIN-M** and **SOY-M** male piglets. The majority of the significant GO terms were linked to pathways involved in the metabolism and biosynthesis of carboxylic acids and polysaccharides, as well as in the regulation and activation of immunological responses. Some of these are as follows: innate immune response (*GO:0045087*), regulation of cytokine-mediated signaling pathway (*GO:0001959*), regulation of alpha-beta T cell activation (*GO:0046634*), B cell differentiation (*GO:0030183*), positive regulation of interleukin-4 production (*GO:0032753*), unsaturated fatty acid biosynthetic process (*GO:0006636*), and glycogen metabolic process (*GO:0005977*).

4.4. n-6/n-3 PUFA ratio and differentially expressed miRNAs and their regulatory functions

Since the discovery of microRNAs (miRNAs), a class of short non-coding RNA molecules with an average length of about 22 nucleotides, the landscape of post-transcriptional regulation of gene expression has substantially changed. miRNAs have the ability to fine-tune the expression of genes linked to a specific metabolic or physiological pathway by base-pairing to specific sequences and binding sites in the 3' untranslated regions (3' UTRs) of targeted mRNAs, where they are able to trigger their degradation and/or inhibit their translation, which further affecting the translation of tens or hundreds of mRNAs into functional proteins. (Bartel, 2018; Filipowicz et al., 2008; Naeli et al., 2022; Ratti et al., 2020).

From our paper on backcross population of Iberian and Duroc pigs, we found four differentially expressed miRNAs (*ssc-miR-30a-3p, ssc-miR-30e-3p, ssc-miR-15b* and *ssc-miR-7142-3p*) between high and low n-6/n-3 PUFA ratio contrast on porcine skeletal muscle. The expression of these DE miRNAs were related to lipid metabolism and immune response. Firstly, the expression of *miR-30a* in pigs has been associated to adipocyte formation, fat deposition, myogenic differentiation, pathogenesis, and immune system (Bai et al., 2014; Hicks et al., 2013; Jiang et al., 2015; Mentzel et al., 2016; Peng et al., 2015; Sarkar et al., 2010; J. Wang et al., 2017). This particular miRNA was also linked to adipogenesis and low-grade chronic inflammation in minipig obesity model and to regulation of porcine *ELOVL* fatty acid elongase 6 (*ELOVL6*) gene that is responsible of FA elongation in the de novo lipogenesis (Corominas et al., 2015). Aside from having the same miRNA seed and belonging to the *miR-30* family, *miR-30e* was also associated to target mRNA genes that are related to porcine skeletal muscle growth, energy metabolism, improved feed conversion rates, pathogenesis, virus-host interactions, and immunomodulation (Bao et al., 2015; Jia et al., 2017; Núñez-Hernández et al., 2017; Ye et al., 2012; Zaragosi et al., 2011).

In addition, *mir-15b* is mainly associated to blood vessel formation (angiogenesis), tumor growth and cellular ATP level modulation and could be related to the n-3-PUFA-

derived substances that contain anti-angiogenic, anti-inflammatory and antitumor properties (Kang & Liu, 2013; Kubiczkova et al., 2012; W. Wang et al., 2014). We have also found a mirton, *ssc-mir-7142*-3p, that has been detected in lung tissue infected with *Actinobacillus pleuropneumoniae* and its differential expression has been associated to the overexpression of the retinol binding protein 4 (*RBP4*) gene, which was related in various pathophysiological processes, such as obesity, insulin resistance and cardiovascular diseases (Podolska et al., 2012; Zhao et al., 2021).

To further demonstrate the miRNA-driven regulation on the DEGs, we have constructed co-expression network by combining relevant miRNA-mRNA expression correlations according to the PCIT algorithm ($r < -0.50$) and 3' UTR region seed matching. We obtained two miRNA-mRNA pairs; (1) *ssc-miR-15b* and arresting domain containing 3 (*ARRDC3*) gene; and (2) *ssc-miR-7142-3p* and methyltransferase-like 21C (*METTL21C*). Aside from its role on on G protein-coupled receptors and β_2-adrenergic receptors, activation of *ARRDC3* could be due to nutrient excess or cellular stressors (Moore et al., 2007; Weinberg & Puthenveedu, 2019). In addition, this gene is also involved in obesity development, insulin resistance, body mass regulation, glucose metabolism, adiposity and energy expenditure (Batista et al., 2020; Ogawa et al., 2019; Patwari et al., 2011). Meanwhile, *METTL21C* is reported to be involved in regulation of myogenesis, muscle function and protein catabolism (C. Wang et al., 2019; Wiederstein et al., 2018). According to our findings, a high concentration of n-3 PUFAs seems to increase the expression of *METTL21C*. This gene's expression was also found to be reduced following prolonged exercise, which was accompanied by increased levels of inflammatory cytokines, oxidative stress, and leukocytosis (Olsen et al., 2021; Wilund, 2007).

The upregulation of *ssc-miR-15b* and downregulation of *ssc-miR-1472-3p*, along with the downregulation of *ARRDC3* and the upregulation of *METTL21C*, in the presence of low n-6/n-3 PUFA ratio, may be related to pro-inflammatory metabolites stimulating receptors related to stress and immunity. However, further validation among these predicted regulatory networks should be done in order to verify their biological importance in terms of porcine growth and immune response.

4.5. n-6/n-3 PUFA ratio and post-transcriptional regulations using EISA

RNA-Seq has been proven as one of the primary methods in providing significant amount of data regarding gene expression levels and has been used, together with canonical DE analysis, to explain some key phenotypic variations among multiple species across various biological conditions (Costa-Silva et al., 2017; Frazee et al., 2014; McDermaid et al., 2019). One limitation of such is the incapacity to account the transcriptional and post-transcriptional aspects of gene expression regulation; distinguished by measuring the changes between the exonic and intronic reads from expressed mRNA transcripts (Gaidatzis et al., 2015; Mármol-Sánchez et al., 2022). Although by comparison, we obtained a different set of DE downregulated genes and those genes showing with strongest post-transcriptional signals. The discrepancies, however, was reduced when we found some common genes between the two analyses employed in this study.

With this, we have also prioritized the genes based on their ΔEx values (at least 1.5-fold reduction) and PTc signal (top 5% negative scores) from the piglets from sows fed with **LIN** and **SOY** diets. EISA demonstrated multiple genes with relevant post-transcriptional downregulation in **LIN-M vs SOY-M** that were linked to various biological functions. The *ST8SIA2* gene displayed one of the most negative PTc scores. *ST8SIA2* gene encodes a type II membrane protein that catalyzes the transfer of sialic acid to N-linked oligosaccharides and glycoproteins (Yang et al., 2022). This function is relevant since studies have shown that a deficiency in sialic acid or N-glycans are associated with oxidative (Cho et al., 2017) and inflammatory stress (Woodward et al., 2019) which could be expected in soy-based diets where the n-6/n-3 PUFA ratio is increased. In spite of its weak signaling, this gene was also found to be targeted by the sole overexpressed miRNA in **SOY-F** piglets. Consequently, we were able to find a group of genes with relevant post-transcriptional signals, suggesting miRNA-driven repression in female pigs, that were related to immunological responses. Given that, the post-transcriptional signals from the female piglets were not as strong and clear as those from the males. Nevertheless, multiple reports have demonstrated sex-biased gene regulation, including transcription factors, which might underline sex-specific regulatory processes in both health and disease (Gershoni & Pietrokovski, 2017; Lopes-Ramos et al., 2020).

Likewise, EISA analysis also provided another relevant gene with high post-transcriptional downregulation such as energy homeostasis associated (*ENHO*). *ENHO* encodes a peptide hormone called adropin, which plays a role in regulating lipid and glucose homeostasis and prevents hyperinsulinemia, dyslipidemia, and impaired glucose tolerance (Es-haghi et al., 2021; Kumar et al., 2008). Adropin deficiency was linked with increased adiposity, insulin resistance, and metabolic defects (Chen et al., 2017; Ganesh-Kumar et al., 2012).

For **SOY-M vs LIN-M** contrast, we have also identified numerous mRNA genes with strong post-transcriptional downregulation and that were also targeted by at least one of the upregulated miRNAs. The gene with the most negative PTc score and has provided binding sites to fifteen upregulated miRNAs was *TMEM71*, a transmembrane protein that is involved in multiple crucial biological processes including immune and inflammatory response (K. Wang et al., 2019). EISA also highlighted several genes that were related to inflammation and immunomodulation. One of which is *EVI2B*, whose functionality also extends to cell proliferation and apoptosis (Yonekura & Ueda, 2021; Zjablovskaja et al., 2017). Arrestin domain containing 2 (*ARRDC2*), as a member of α-arrestins, play a vital role in G-protein-coupled receptor signaling and cancer progression; malfunction of which could lead to diabetes, obesity, cardiovascular diseases (Zalewska et al., n.d.). Our results also showed many cluster of differentiation (*CD*) molecules (i.e., *CD3ε*, *CD7*, *CD22*, *CD48*, *CD68*, *CD86*, *CD163*) that were frequently corresponded to cytokine production, T-cell regulation, immune response, and/or inflammation (Clark & Giltiay, 2018; Lv et al., 2021; McArdel et al., 2016; Siwan et al., 2022; Soudais et al., 1993; Sun et al., 2019; Zeibig et al., 2019) and some were now reported to be linked with miRNAs for immunotherapy use in cancer (Zhang et al., 2021).

We have also identified a few genes that were associated with, but not limited to, lipid metabolism, lipoprotein metabolism, atherosclerosis, obesity and/or inflammatory regulation: porcine-specific cytochrome P450 family 3 subfamily A member 22 (*CYP3A22*) (Konkel & Schunck, 2011); cadherin related family member 1 (*CDHR1*); and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (*PIK3CD*) (Ballas, 2018; Virk et al., 2022).

Furthermore, our heatmaps from our paper have demonstrated the correlation between genes, miRNAs, and the analyzed PUFAs on SOY and LIN piglets. Conversely, we observed four genes that were supposedly post-transcriptionally downregulated by upregulated miRNAs after EISA analysis, but further displayed direct correlation. These genes (i.e., *COL22A1*, *ALB*, *LIPG*, and *ADIPOQ*) were associated in lipid-related and lipid-mediated functions and pathways. The detected post-transcriptional repression might be induced by other post-transcriptional effectors, like PUFAs, that could be acting on their transcription factors and altering their expression (Clarke, 2001; Sessler & Ntambi, 1998). In addition, considering that the PUFAs employed in the experiment have antagonistic effects and may produce metabolites that might regulate one another, genetic differences and the animal's age may potentially affect the expression of protein-coding genes and miRNAs (Huan et al., 2018; Schmitz & Ecker, 2008). These intrinsic factors and nutritional interventions have the possibility to modulate overall expression profile of mRNA and miRNAs (Butler, 2014). Even though majority of which were not captured to be downregulated after canonical DE analysis, EISA could serve as a complimentary approach to provide changes in gene expression, coupled with miRNA-driven suppression, at the exonic and intronic level. Likewise, our analyses demonstrated a number of genes that were primarily involved in the modulation of lipid-related functions and immune responses, which may be linked to the antagonistic inflammatory actions of the n-6 and n-3 PUFAs.

4.6. n-6/n-3 PUFA ratio and differentially expressed proteins and their relative abundance

We have also employed the use of proteomics in determining proteins and their relative abundance, in response to **SOY** and **LIN** diets, on porcine skeletal muscle and adipose tissues. Proteome Discoverer 2.5 software allowed us to determine 4 and 11 differentially expressed proteins in SOY over LIN from *longissimus dorsi* muscle and subcutaneous fat, respectively. The overabundance of muscle and adipose proteins observed with SOY diets primarily demonstrated relationship with immune response, fatty

acid metabolism, glycolysis and gluconeogenesis, and inflammatory response pathways. Due to antagonistic inflammatory function between n-6 and n-3 PUFAs, an increased supply of SOY in diets may trigger the pro-inflammatory function and effects of n-6 PUFA. While majority of eicosanoids are produced from n-6 PUFA AA, some arise from processing of n-3 PUFAs such as EPA or DHA. Eicosanoid signaling, as with cytokine signaling and inflammasome formation, has been tagged as a primary pro-inflammatory component of innate immunity (Dennis & Norris, 2015; Lone & Taskén, 2013).

One of the overabundant proteins that were both found in muscle and adipose tissue was haptoglobin, in which its increase relative abundance could be related to the pro-inflammatory function of n-6 PUFA. Haptoglobin is classified and is considered as one of the main acute-phase proteins in pigs. This plasma protein is used as a diagnostic tool to assess diseases, health status, and production performance. As it is mostly synthesized in the liver and dependent on the synthesis of interleukin 6 (*IL-6*), it also protects the host against the dangers of acute-phase reactions (Dobryszycka, 1997; Eurell et al., 1992; Petersen et al., 2002). Generally, if the animals are subjected to health or stress-related challenges, they activate both their innate and immune systems and signal their defense mechanisms and pathophysiological responses (i.e., antibodies production, leukocyte activity, acute-phase protein production) (Jain et al., 2011). In human studies, haptoglobin is considered as an indicator of obesity. It is expressed by adipocytes and its abundance in white adipose tissue and in plasma shows a direct correlation with the degree of adiposity (Fain et al., 2004; Maffei et al., 2016). Serum levels of haptoglobin were elevated during weaning, which indicates exposure to stress or inflammatory stimuli (Sauerwein et al., 2005). To our knowledge, this is the first study that detects haptoglobin in muscle and adipose tissue in pre-weaning piglets.

Genes associated with the glycolysis and gluconeogenesis pathway may have been activated as a result of higher dietary amounts of n-6 PUFA. *PGK2* encodes the protein that is responsible for the first catalytic ATP-generating step in the glycolytic pathway. On the other hand, dietary PUFAs (such as LA, EPA, and DHA) are known to be powerful metabolic enzyme inhibitors that can silence glycolytic and lipogenic genes (Dentin, 2005; Yao et al., 2006). Another common protein that was found is liver carboxylesterase, an

enzyme is found in the liver and further protects the organ from alcohol- or diet-induced inflammation, damage, or injury (Xu et al., 2016). Moreover, **SOY** also stimulated protegrins, a type of cathelicidins which are part of the innate immune system and has antimicrobial capacity against Gram-positive and Gram-negative bacteria and reported to possess immunomodulatory functions (Harwig et al., 1996; Scheenstra et al., 2020).

Based on our results, we have also identified overabundant proteins that were related to responses to viral infections, lipid peroxidation, atherosclerosis, glucose oxidation, and inflammation. These findings could still be associated to the pro-inflammatory functions and lipid-related responses of n-6 PUFA.

4.7. Future perspectives and challenges

The application of omics-based technologies (genomics, transcriptomics, and proteomics) has allowed the identification of genes, miRNAs, related biological pathways, post-transcriptional regulation, and overabundant proteins in response to n-6/n-3 PUFA ratio. These identified molecular markers were mostly tissue-specific (i.e., skeletal muscle or adipose tissue), and associated to physiological responses and regulation related to lipid metabolism, cell proliferation, innate immune response and inflammation. These responses could be associated to the antagonistic inflammatory functions of n-6 and n-3 PUFAs, or even so, to the pro-inflammatory property of SOY as some of the differentially expressed genes, miRNAs, and proteins were linked to immunity and immunomodulation; signaling cellular stress or infection.

Furthermore, as the trend of over-supplementation of n-6 PUFAs has becoming apparent in commercial swine diets, our research could show on how the ingestion of such, starting from the gestation, could be transferred to sow's colostrum and milk, and be incorporated into suckling piglets. Furthermore, this could also affect the post-weaning growth and entire production performance of the animal. Optimizing, or rather, lowering the ratio between n-6 and n-3 in swine diets could be beneficial for both sow and piglets.

OMICs approaches used in our dissertation have outline some relevant information regarding changes in immune-related functions of pigs in response to high or low n-6/n-3 PUFA ratios, but further studies including liver and serum could complement these findings and develop a more in-depth picture on how the different ratios between n-6 and n-3 PUFA influence the overall performance and health status of the animal.

CONCLUSIONS

Chapter 5

1. According to differences in n-6/n-3 PUFA ratio, the transcriptome analysis of 20 Iberian x Duroc *longissimus dorsi* muscle samples highlighted 432 differentially expressed genes and enriched pathways related to lipid metabolism, cell growth, and inflammation, together with 4 differentially expressed microRNAs (*ssc-miR-30a-3p, ssc-miR-30e-3p, ssc-miR-15b* and *ssc-miR-7142-3p*) that were associated to adipogenesis and immunity.

2. Relevant miRNA-to-mRNA regulatory networks associated with changes in the n-6/n-3 ratio in porcine *longissimus dorsi* muscle were unveiled (i.e., *mir15b* to *ARRDC3*; *mir-7142-3p* to *METTL21C*). These co-expressions between mRNA genes and miRNAs were further linked to lipolysis, obesity, myogenesis, and protein degradation, whereas putative mRNA-to-mRNA correlations emphasized genes related to lipid-mediated biological processes and immunity.

3. The implementation of extreme n-6/n-3 PUFA ratios of 13:1 (SOY) and 4:1 (LIN) on gestation and lactation diets of sows influenced their reproductive performance and pre-weaning growth performance of piglets. LIN decreased dead-born piglets and pre-weaning mortality by 22.50% compared to SOY. Higher individual body weight was detected in LIN piglets on day 15 of lactation, but no differences between LIN and SOY were observed at birth, weaning, or overall weight gain of piglets.

4. Fatty acid composition of muscle and adipose tissues showed great resemblance to the dietary treatments and the same trend for total n-3, n-6, and n-6/n-3 ratios. A lower n-6/n-3 ratio in sow diets was reflected in a lower n-6/n-3 LD muscle and adipose tissue content in piglets, mainly driven by the increase of n-3 PUFAs in both tissues.

5. Proteomics analysis revealed 4 and 11 overabundant proteins in muscle and adipose tissues in SOY compared to LIN diets, respectively. These proteins were primarily

linked with pathways related to immune response, fatty acid metabolism, glycolysis and gluconeogenesis, and the inflammatory response. Four common overabundant proteins were found in both muscle and adipose tissue samples (i.e., haptoglobin, phosphoglycerate kinase 2, liver carboxylesterase, and porcine antimicrobial peptides protegrin). The overexpression of the acute-phase protein haptoglobin in SOY, as well as the activation of genes and proteins involved in the innate immune response and acute inflammatory response, may be linked to the pro-inflammatory effects of n-6 PUFAs.

6. Exon-intron split analysis (EISA) approach was used to take into consideration post-transcriptional modifications induced by potential functional miRNA repression in the *longissimus dorsi* muscle of male and female piglets, in response to extreme n-6/n-3 PUFA ratios. The use of EISA revealed 11 and 97 mRNA genes within the top 5% negative PTc scores with at least 1.5-fold exonic region reduction in SOY-male (SOY-M) and LIN-male (LIN-M) piglets, respectively. Furthermore, the detected upregulated miRNAs harbored binding sites on 72.73% (SOY-M) and 61.86% (LIN-M) of these post-transcriptionally downregulated genes. Although we detected 30 post-transcriptionally downregulated mRNA genes and one upregulated miRNA in SOY-female piglets, the overall PTc signals in females were not as strong and clear as those from the males.

7. *ST8SIA2* (SOY-M and SOY-F) and *TMEM71* (LIN-M) are two of the genes with the most negative PTc scores; *ST8SIA2* is associated with oxidative and inflammatory stress, whereas *TMEM71* encodes a transmembrane protein that is involved in immune and inflammatory response. Likewise, we discovered genes that are predominantly associated in immunomodulation and lipid-related metabolic processes, which could be due to the pro- and anti-inflammatory properties of n-6 and n-3 PUFAs, respectively.

8. PUFA abundance in porcine *longissimus dorsi* muscle was correlated with the expression of post-transcriptionally downregulated mRNAs and overexpressed miRNAs. Our results highlighted ALA (n-3) and LA (n-6) as two of the main drivers of the overall phenotypic variance observed in response to dietary treatments.

9. We also detected mRNA genes that were post-transcriptionally repressed and significantly downregulated after differential expression analysis but were not targeted by any upregulated miRNAs (i.e., ALB and COL22A1 in SOY-M piglets; *LIPG* and *ADIPOQ* in LIM-M piglets). Even though miRNAs play a key role in post-transcriptional regulation, additional post-transcriptional effectors including long non-coding RNAs, circular RNAs, or RNA binding proteins may also be involved.

10. The implementation of EISA in detecting post-transcriptionally downregulated genes allowed us to uncover co-expression networks among differentially expressed miRNAs and their targeted mRNAs at the post-transcriptional level, thus complementing canonical differential expression analyses and providing a more comprehensive view of miRNA-driven regulation of mRNA genes and metabolic changes in response to n-6/n-3 PUFA ratio.

REFERENCES

Chapter 6

Aaslyng, M. D., Oksama, M., Olsen, E. V., Bejerholm, C., Baltzer, M., Andersen, G., Bredie, W. L. P., Byrne, D. V., & Gabrielsen, G. (2007). The impact of sensory quality of pork on consumer preference. *Meat Science*, *76*(1), 61–73. https://doi.org/10.1016/j.meatsci.2006.10.014

Amusquivar, E., Laws, J., Clarke, L., & Herrera, E. (2010). Fatty acid composition of the maternal diet during the first or the second half of gestation influences the fatty acid composition of sows' milk and plasma, and plasma of their piglets. *Lipids*, *45*(5), 409–418. https://doi.org/10.1007/s11745-010-3415-2

Archibald, A. L., Haley, C. S., Brown, J. F., Couperwhite, S., McQueen, H. A., Nicholson, D., Coppieters, W., Van de Weghe, A., Stratil, A., Winterø, A. K., Fredholm, M., Larsen, N. J., Nielsen, V. H., Milan, D., Woloszyn, N., Robic, A., Dalens, M., Riquet, J., Gellin, J., … Schmitz, C. B. (1995). The PiGMaP consortium linkage map of the pig (Sus scrofa). *Mammalian Genome*, *6*(3), 157–175. https://doi.org/10.1007/BF00293008

Archibald, A. L., Bolund, L., Churcher, C., Fredholm, M., Groenen, M. A. M., Harlizius, B., Lee, K.-T., Milan, D., Rogers, J., Rothschild, M. F., Uenishi, H., Wang, J., Schook, L. B., & Genome Sequencing Consortium, S. (2010). Pig genome sequence - analysis and publication strategy. *BMC Genomics*, *11*(1), 438. https://doi.org/10.1186/1471-2164-11-438

Bai, Y., Huang, J.-M., Liu, G., Zhang, J.-B., Wang, J.-Y., Liu, C.-K., & Fang, M.-Y. (2014). A comprehensive microRNA expression profile of the backfat tissue from castrated and intact full-sib pair male pigs. *BMC Genomics*, *15*(1), 47. https://doi.org/10.1186/1471-2164-15-47

Ballas, Z. K. (2018). The Editors' Choice. *Journal of Allergy and Clinical Immunology*, *142*(2), 394–395. https://doi.org/10.1016/j.jaci.2018.06.028

Bao, H., Kommadath, A., Liang, G., Sun, X., Arantes, A. S., Tuggle, C. K., Bearson, S. M. D., Plastow, G. S., Stothard, P., & Guan, L. L. (2015). Genome-wide whole blood microRNAome and transcriptome analyses reveal miRNA-mRNA regulated host response to foodborne pathogen Salmonella infection in swine. *Scientific Reports*, *5*(1), 12620. https://doi.org/10.1038/srep12620

Bartel, D. P. (2004). MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell*, *116*(2), 281–297. https://doi.org/10.1016/S0092-8674(04)00045-5

Bartel, D. P. (2018). Metazoan MicroRNAs. *Cell*, *173*(1), 20–51. https://doi.org/10.1016/j.cell.2018.03.006

Batista, T. M., Dagdeviren, S., Carroll, S. H., Cai, W., Melnik, V. Y., Noh, H. L., Saengnipanthkul, S., Kim, J. K., Kahn, C. R., & Lee, R. T. (2020). Arrestin domain-containing 3 (Arrdc3) modulates insulin action and glucose metabolism in liver. *Proceedings of the National Academy of Sciences*, *117*(12), 6733–6740. https://doi.org/10.1073/pnas.1922370117

Bejerholm, A. C., & Barton-Gade, P. (1986). Effect of intramuscular fat level on eating quality of pig meat. *32nd International Congress of Meat Science and Techonology*,

389–391.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. https://doi.org/10.1139/o59-099

Brewer, M. S., Zhu, L. G., & McKeith, F. K. (2001). Marbling effects on quality characteristics of pork loin chops: Consumer purchase intent, visual and sensory characteristics. *Meat Science*, *59*(2), 153–163. https://doi.org/10.1016/S0309-1740(01)00065-1

Butler, G. (2014). Manipulating dietary PUFA in animal feed: implications for human health. *Proceedings of the Nutrition Society*, *73*(1), 87–95. https://doi.org/10.1017/S0029665113003790

Cai, Y., Yu, X., Hu, S., & Yu, J. (2009). A Brief Review on the Mechanisms of miRNA Regulation. *Genomics, Proteomics and Bioinformatics*, *7*(4), 147–154. https://doi.org/10.1016/S1672-0229(08)60044-3

Carlson, S. E., Werkman, S. H., Peeples, J. M., Cooke, R. J., & Tolley, E. A. (1993). Arachidonic acid status correlates with first year growth in preterm infants. *Proceedings of the National Academy of Sciences*, *90*(3), 1073–1077. https://doi.org/10.1073/pnas.90.3.1073

Chen, S., Zeng, K., Liu, Q., Guo, Z., Zhang, S., Chen, X., Lin, J., Wen, J., Zhao, C., Lin, X., & Gao, F. (2017). Adropin deficiency worsens HFD-induced metabolic defects. *Cell Death & Disease*, *8*(8), e3008–e3008. https://doi.org/10.1038/cddis.2017.362

Chilliard, Y. (1993). Dietary fat and adipose tissue metabolism in ruminants, pigs, and rodents: a review. *Journal of Dairy Science*, *76*(12), 3897–3931. https://doi.org/10.3168/jds.S0022-0302(93)77730-9

Cho, A., Christine, M., Malicdan, V., Miyakawa, M., Nonaka, I., Nishino, I., & Noguchi, S. (2017). Sialic acid deficiency is associated with oxidative stress leading to muscle atrophy and weakness in GNE myopathy. *Human Molecular Genetics*, *26*(16), 3081–3093. https://doi.org/10.1093/hmg/ddx192

Choi, J.-S., Lee, H.-J., Jin, S.-K., Choi, Y.-I., & Lee, J.-J. (2014). Comparison of carcass characteristics and meat quality between Duroc and crossbred pigs. *Korean Journal for Food Science of Animal Resources*, *34*(2), 238–244. https://doi.org/10.5851/kosfa.2014.34.2.238

Clark, E. A., & Giltiay, N. V. (2018). CD22: A Regulator of Innate and Adaptive B Cell Responses and Autoimmunity. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.02235

Clarke, S. D. (2001). Polyunsaturated Fatty Acid Regulation of Gene Transcription: A Molecular Mechanism to Improve the Metabolic Syndrome. *The Journal of Nutrition*, *131*(4), 1129–1132. https://doi.org/10.1093/jn/131.4.1129

Corino, C., Rossi, R., Cannata, S., & Ratti, S. (2014). Effect of dietary linseed on the nutritional value and quality of pork and pork products: Systematic review and meta-

analysis. *Meat Science*, *98*(4), 679–688. https://doi.org/10.1016/j.meatsci.2014.06.041

Corominas, J., Marchesi, J. A., Puig-Oliveras, A., Revilla, M., Estellé, J., Alves, E., Folch, J. M., & Ballester, M. (2015). Epigenetic regulation of the ELOVL6 gene is associated with a major QTL effect on fatty acid composition in pigs. *Genetics Selection Evolution*, *47*(1), 20. https://doi.org/10.1186/s12711-015-0111-y

Costa-Silva, J., Domingues, D., & Lopes, F. M. (2017). RNA-Seq differential expression analysis: An extended review and a software tool. *PLOS ONE*, *12*(12), e0190152. https://doi.org/10.1371/journal.pone.0190152

Daszkiewicz, T., Bak, T., & Denaburski, J. (2005). Quality of pork with a different intramuscular fat (IMF) content. *Polish Journal of Food and Nutrition Sciences*, *14*(1), 31–35.

De la Llata, M., Dritz, S. S., Tokach, M. D., Goodband, R. D., Nelssen, J. L., & Loughin, T. M. (2001). Effects of dietary fat on growth performance and carcass characteristics of growing-finishing pigs reared in a commercial environment. *Journal of Animal Science*, *79*(10), 2643. https://doi.org/10.2527/2001.79102643x

DeBose-Boyd, R. A., & Ye, J. (2018). SREBPs in Lipid Metabolism, Insulin Signaling, and Beyond. *Trends in Biochemical Sciences*, *43*(5), 358–368. https://doi.org/10.1016/j.tibs.2018.01.005

Dennis, E. A., & Norris, P. C. (2015). Eicosanoid storm in infection and inflammation. *Nature Reviews Immunology*, *15*(8), 511–523. https://doi.org/10.1038/nri3859

Dentin, R. (2005). Polyunsaturated fatty acids suppress glycolytic and lipogenic genes through the inhibition of ChREBP nuclear protein translocation. *Journal of Clinical Investigation*, *115*(10), 2843–2854. https://doi.org/10.1172/JCI25256

DeRouchey, J. M., Dritz, S. S., Goodband, R. D., Nelssen, J. L., & Tokach, M. D. (2007). *KSU Swine Nutrition Guide - General Nutrition Principles for Swine*. Kansas State University Agricultural Experiment Station and Cooperative Extension Service. https://bookstore.ksre.ksu.edu/pubs/mf2298.pdf

Díaz, M., García, C., Sebastiani, G., De Zegher, F., López-Bermejo, A., & Ibáñez, L. (2017). Placental and cord blood methylation of genes involved in energy homeostasis: Association with fetal growth and neonatal body composition. *Diabetes*, *66*(3), 779–784. https://doi.org/10.2337/db16-0776

Dobryszycka, W. (1997). Biological functions of haptoglobin-new pieces to an old puzzle. *Clinical Chemistry and Laboratory Medicine*, *35*(9), 647–654. https://doi.org/10.1515/cclm.1997.35.9.647

Duan, Y., Li, F., Li, L., Fan, J., Sun, X., & Yin, Y. (2014). n-6:n-3 PUFA ratio is involved in regulating lipid metabolism and inflammation in pigs. *British Journal of Nutrition*, *111*(3), 445–451. https://doi.org/10.1017/S0007114513002584

Duncan, R. E., Ahmadian, M., Jaworski, K., Sarkadi-Nagy, E., & Sul, H. S. (2007). Regulation of Lipolysis in Adipocytes. *Annual Review of Nutrition*, *27*(1), 79–101.

https://doi.org/10.1146/annurev.nutr.27.061406.093734

EMBL-EBI. (2019). *What is Next Generation DNA Sequencing?* https://www.ebi.ac.uk/training/online/courses/functional-genomics-ii-common-technologies-and-data-analysis-methods/next-generation-sequencing/

Ernst, C. W., & Steibel, J. P. (2013). Molecular advances in QTL discovery and application in pig breeding. *Trends in Genetics*, *29*(4), 215–224. https://doi.org/10.1016/j.tig.2013.02.002

Es-haghi, A., Al-Abyadh, T., & Mehrad-Majd, H. (2021). The Clinical Value of Serum Adropin Level in Early Detection of Diabetic Nephropathy. *Kidney and Blood Pressure Research*, *46*(6), 734–740. https://doi.org/10.1159/000519173

Eurell, T. E., Bane, D. P., Hall, W. F., & Schaeffer, D. J. (1992). Serum haptoglobin concentration as an indicator of weight gain in pigs. *Canadian Journal of Veterinary Research = Revue Canadienne de Recherche Veterinaire*, *56*(1), 6–9. http://www.ncbi.nlm.nih.gov/pubmed/1586895

Fain, J. N., Bahouth, S. W., & Madan, A. K. (2004). Haptoglobin release by human adipose tissue in primary culture. *Journal of Lipid Research*, *45*(3), 536–542. https://doi.org/10.1194/jlr.M300406-JLR200

Fang, X., Mou, Y., Huang, Z., Li, Y., Han, L., Zhang, Y., Feng, Y., Chen, Y., Jiang, X., Zhao, W., Sun, X., Xiong, Z., Yang, L., Liu, H., Fan, D., Mao, L., Ren, L., Liu, C., Wang, J., … Du, Y. (2012). The sequence and analysis of a Chinese pig genome. *GigaScience*, *1*(1), 16. https://doi.org/10.1186/2047-217X-1-16

Farnworth, E. R., & Kramer, J. K. G. (1987). Fat metabolism in growing swine: a review. *Canadian Journal of Animal Science*, *67*(2), 301–318. https://doi.org/10.4141/cjas87-029

Fernandez, X., Monin, G., Talmant, A., Mourot, J., & Lebret, B. (1999). Influence of intramuscular fat content on the quality of pig meat — 1. Consumer acceptability of m. longissimus lumborum. *Meat Science*, *53*(1), 67–72. https://doi.org/10.1016/S0309-1740(99)00038-8

Filipowicz, W., Bhattacharyya, S. N., & Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nature Reviews Genetics*, *9*(2), 102–114. https://doi.org/10.1038/nrg2290

Folch, J., Lees, M., & Stanley, G. H. S. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry*, *226*(1), 497–509. https://doi.org/10.1016/S0021-9258(18)64849-5

Food and Agriculture Organization (FAO). (2022). *Meat Market Review - Overview of market and policy developments in 2021*. https://www.fao.org/3/cc0984en/cc0984en.pdf

Food and Agriculture Organization of the United Nations (FAO). (2021). *Crop and livestock products - world total meat production quantity*. FAOSTAT. https://www.fao.org/faostat/en/#data/QCL

Frantz, L., Meijaard, E., Gongora, J., Haile, J., Groenen, M. A. M., & Larson, G. (2016). The Evolution of Suidae. *Annual Review of Animal Biosciences*, *4*(1), 61–85. https://doi.org/10.1146/annurev-animal-021815-111155

Frazee, A. C., Sabunciyan, S., Hansen, K. D., Irizarry, R. A., & Leek, J. T. (2014). Differential expression analysis of RNA-seq data at single-base resolution. *Biostatistics*, *15*(3), 413–426. https://doi.org/10.1093/biostatistics/kxt053

Freeman, C. P. (1983). Fat supplementation in animal production—monogastric animals. *Proceedings of the Nutrition Society*, *42*(2), 351–359. https://doi.org/10.1079/PNS19830038

Fukunaga, R. (2016). miRNAs/Small Noncoding RNAs. In *Encyclopedia of Cell Biology* (Vol. 1). Elsevier Ltd. https://doi.org/10.1016/B978-0-12-394447-4.10055-0

Gaidatzis, D., Burger, L., Florescu, M., & Stadler, M. B. (2015). Analysis of intronic and exonic reads in RNA-seq data characterizes transcriptional and post-transcriptional regulation. *Nature Biotechnology*, *33*(7), 722–729. https://doi.org/10.1038/nbt.3269

Ganesh-Kumar, K., Zhang, J., Gao, S., Rossi, J., McGuinness, O. P., Halem, H. H., Culler, M. D., Mynatt, R. L., & Butler, A. A. (2012). Adropin Deficiency Is Associated With Increased Adiposity and Insulin Resistance. *Obesity*, *20*(7), 1394–1402. https://doi.org/10.1038/oby.2012.31

Gebert, L. F. R., & MacRae, I. J. (2019). Regulation of microRNA function in animals. *Nature Reviews Molecular Cell Biology*, *20*(1), 21–37. https://doi.org/10.1038/s41580-018-0045-7

Georgiadi, A., & Kersten, S. (2012). Mechanisms of Gene Regulation by Fatty Acids. *Advances in Nutrition*, *3*(2), 127–134. https://doi.org/10.3945/an.111.001602

Gershoni, M., & Pietrokovski, S. (2017). The landscape of sex-differential transcriptome and its consequent selection in human adults. *BMC Biology*, *15*(1), 7. https://doi.org/10.1186/s12915-017-0352-z

Gjerlaug-Enger, E., Haug, A., Gaarder, M., Ljøkjel, K., Stenseth, R. S., Sigfridson, K., Egelandsdal, B., Saarem, K., & Berg, P. (2015). Pig feeds rich in rapeseed products and organic selenium increased omega-3 fatty acids and selenium in pork meat and backfat. *Food Science & Nutrition*, *3*(2), 120–128. https://doi.org/10.1002/fsn3.182

Govindarajan, R., Duraiyan, J., Kaliyappan, K., & Palanisamy, M. (2012). Microarray and its applications. *Journal of Pharmacy and Bioallied Sciences*, *4*(6), 310. https://doi.org/10.4103/0975-7406.100283

Groenen, M. A. M. (2016). A decade of pig genome sequencing: a window on pig domestication and evolution. *Genetics Selection Evolution*, *48*(1), 23. https://doi.org/10.1186/s12711-016-0204-2

Groenen, M. A. M., Archibald, A. L., Uenishi, H., Tuggle, C. K., Takeuchi, Y., Rothschild, M. F., Rogel-Gaillard, C., Park, C., Milan, D., Megens, H.-J., Li, S., Larkin, D. M., Kim, H., Frantz, L. A. F., Caccamo, M., Ahn, H., Aken, B. L., Anselmo, A., Anthon, C., … Schook, L. B. (2012). Analyses of pig genomes provide insight into porcine

demography and evolution. *Nature*, *491*(7424), 393–398. https://doi.org/10.1038/nature11622

Hamilton, D. N., Ellis, M., Miller, K. D., McKeith, F. K., & Parrett, D. F. (2000). The effect of the Halothane and Rendement Napole genes on carcass and meat quality characteristics of pigs. *Journal of Animal Science*, *78*(11), 2862–2867. https://doi.org/10.2527/2000.78112862x

Harwig, S. S. L., Waring, A., Yang, H. J., Cho, Y., Tan, L., & Lehrer, R. I. (1996). Intramolecular disulfide bonds enhance the antimicrobial and lytic activities of protegrins at physiological sodium chloride concentrations. *European Journal of Biochemistry*, *240*(2), 352–357. https://doi.org/10.1111/j.1432-1033.1996.0352h.x

Hicks, J. A., Yoo, D., & Liu, H.-C. (2013). Characterization of the microRNAome in Porcine Reproductive and Respiratory Syndrome Virus Infected Macrophages. *PLoS ONE*, *8*(12), e82054. https://doi.org/10.1371/journal.pone.0082054

Hocquette, J. F., Gondret, F., Baza, E., Mdale, F., Jurie, C., & Pethick, D. W. (2010). Intramuscular fat content in meat-producing animals: Development, genetic and nutritional control, and identification of putative markers. *Animal*, *4*(2), 303–319. https://doi.org/10.1017/S1751731109991091

Horgan, R. P., & Kenny, L. C. (2011). 'Omic' technologies: genomics, transcriptomics, proteomics and metabolomics. *The Obstetrician & Gynaecologist*, *13*(3), 189–195. https://doi.org/10.1576/toag.13.3.189.27672

Houghton, P. L., & Turlington, L. M. (1992). Application of ultrasound for feeding and finishing animals: a review. *Journal of Animal Science*, *70*(3), 930–941. https://doi.org/10.2527/1992.703930x

Hu, Z. L., Park, C. A., & Reecy, J. M. (2019). Building a livestock genetic and genomic information knowledgebase through integrative developments of Animal QTLdb and CorrDB. *Nucleic Acids Research*, *47*(D1), D701–D710. https://doi.org/10.1093/nar/gky1084

Hu, Z., Wang, Z., & Xu, S. (2012). An Infinitesimal Model for Quantitative Trait Genomic Value Prediction. *PLoS ONE*, *7*(7), e41336. https://doi.org/10.1371/journal.pone.0041336

Huan, T., Chen, G., Liu, C., Bhattacharya, A., Rong, J., Chen, B. H., Seshadri, S., Tanriverdi, K., Freedman, J. E., Larson, M. G., Murabito, J. M., & Levy, D. (2018). Age-associated microRNA expression in human peripheral blood is associated with all-cause mortality and age-related traits. *Aging Cell*, *17*(1), e12687. https://doi.org/10.1111/acel.12687

Humphray, S. J., Scott, C. E., Clark, R., Marron, B., Bender, C., Camm, N., Davis, J., Jenks, A., Noon, A., Patel, M., Sehra, H., Yang, F., Rogatcheva, M. B., Milan, D., Chardon, P., Rohrer, G., Nonneman, D., de Jong, P., Meyers, S. N., … Rogers, J. (2007). A high utility integrated map of the pig genome. *Genome Biology*, *8*(7), R139. https://doi.org/10.1186/gb-2007-8-7-r139

Jain, S., Gautam, V., & Naseem, S. (2011). Acute-phase proteins: As diagnostic tool.

Journal of Pharmacy and Bioallied Sciences, *3*(1), 118. https://doi.org/10.4103/0975-7406.76489

Jamuar, S. S., D'Gama, A. M., & Walsh, C. A. (2016). *Genomics, Circuits, and Pathways in Clinical Neuropsychiatry* (T. Lehner, B. L. Miller, & M. W. State (eds.); 1st editio). Elsevier. https://doi.org/10.1016/C2013-0-13583-0

Jazayeri, S. M., Melgarejo-Muñoz, L. M., & Romero, H. M. (2014). RNA-Seq: A glance at technologies and methodologies. *Acta Biológica Colombiana*, *20*(2), 23–35. https://doi.org/10.15446/abc.v20n2.43639

Jia, H., Zhao, Y., Li, T., Zhang, Y., & Zhu, D. (2017). miR-30e is negatively regulated by myostatin in skeletal muscle and is functionally related to fiber-type composition. *Acta Biochimica et Biophysica Sinica*, *49*(5), 392–399. https://doi.org/10.1093/abbs/gmx019

Jiang, P., Zhou, N., Chen, X., Zhao, X., Li, D., Wang, F., Bi, L., & Zhang, D. (2015). Integrative analysis of differentially expressed microRNAs of pulmonary alveolar macrophages from piglets during H1N1 swine influenza A virus infection. *Scientific Reports*, *5*(1), 8167. https://doi.org/10.1038/srep08167

Jin, Y., Liu, S., Yuan, Z., Yang, Y., Tan, S., & Liu, Z. (2016). Catfish genomic studies: Progress and perspectives. In *Genomics in Aquaculture*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-801418-9.00004-4

Jones, M. L., Mark, P. J., Mori, T. A., Keelan, J. A., & Waddell, B. J. (2013). Maternal dietary omega-3 fatty acid supplementation reduces placental oxidative stress and increases fetal and placental growth in the rat. *Biology of Reproduction*, *88*(2), 1–8. https://doi.org/10.1095/biolreprod.112.103754

Jump, D. B. (2011). Fatty acid regulation of hepatic lipid metabolism. *Current Opinion in Clinical Nutrition and Metabolic Care*, *14*(2), 115–120. https://doi.org/10.1097/MCO.0b013e328342991c

Jung, H. J., Kim, Y. Y., & Han, I. K. (2003). Effects of Fat Sources on Growth Performance, Nutrient Digestibility, Serum Traits and Intestinal Morphology in Weaning Pigs. *Asian-Australasian Journal of Animal Sciences*, *16*(7), 1035–1040. https://doi.org/10.5713/ajas.2003.1035

Kang, J. X., & Liu, A. (2013). The role of the tissue omega-6/omega-3 fatty acid ratio in regulating tumor angiogenesis. *Cancer and Metastasis Reviews*, *32*(1–2), 201–210. https://doi.org/10.1007/s10555-012-9401-9

Karahalil, B. (2016). Overview of Systems Biology and Omics Technologies. *Current Medicinal Chemistry*, *23*(37), 4221–4230. https://doi.org/10.2174/0929867323666160926150617

Kaur, N., Chugh, V., & Gupta, A. K. (2014). Essential fatty acids as functional components of foods- a review. *Journal of Food Science and Technology*, *51*(10), 2289–2303. https://doi.org/10.1007/s13197-012-0677-0

Kil, D., & Stein, H. (2011). Dietary soybean oil and choice white grease improve apparent

ileal digestibility of amino acids in swine diets containing corn, soybean meal, and distillers dried grains with soluble. *Rev Colomb Cienc Pecu*, *24*, 248–253. https://www.redalyc.org/pdf/2950/295022382003.pdf

Kloareg, M., Noblet, J., & van Milgen, J. (2007). Deposition of dietary fatty acids, de novo synthesis and anatomical partitioning of fatty acids in finishing pigs. *British Journal of Nutrition*, *97*(1), 35–44. https://doi.org/10.1017/S0007114507205793

Konkel, A., & Schunck, W.-H. (2011). Role of cytochrome P450 enzymes in the bioactivation of polyunsaturated fatty acids. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1814*(1), 210–222. https://doi.org/10.1016/j.bbapap.2010.09.009

Kouba, M., Enser, M., Whittington, F. M., Nute, G. R., & Wood, J. D. (2003). Effect of a high-linolenic acid diet on lipogenic enzyme activities, fatty acid composition, and meat quality in the growing pig1. *Journal of Animal Science*, *81*(8), 1967–1979. https://doi.org/10.2527/2003.8181967x

Kubiczkova, L., Sedlarikova, L., Hajek, R., & Sevcikova, S. (2012). TGF-β – an excellent servant but a bad master. *Journal of Translational Medicine*, *10*(1), 183. https://doi.org/10.1186/1479-5876-10-183

Kumar, K. G., Trevaskis, J. L., Lam, D. D., Sutton, G. M., Koza, R. A., Chouljenko, V. N., Kousoulas, K. G., Rogers, P. M., Kesterson, R. A., Thearle, M., Ferrante, A. W., Mynatt, R. L., Burris, T. P., Dong, J. Z., Halem, H. A., Culler, M. D., Heisler, L. K., Stephens, J. M., & Butler, A. A. (2008). Identification of Adropin as a Secreted Factor Linking Dietary Macronutrient Intake with Energy Homeostasis and Lipid Metabolism. *Cell Metabolism*, *8*(6), 468–481. https://doi.org/10.1016/j.cmet.2008.10.011

Laliotis, G. P., Bizelis, I., & Rogdakis, E. (2010). Comparative approach of the de novo fatty acid synthesis (lipogenesis) between ruminant and non ruminant mammalian species: from biochemical level to the main regulatory lipogenic genes. *Current Genomics*, *11*(3), 168–183. https://doi.org/10.2174/138920210791110960

Lander, E. S., & Botstein, D. (1989). Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics*, *121*(1), 185–199. https://doi.org/10.1093/genetics/121.1.185

Lanktree, M. B., Joy, T. R., & Hegele, R. A. (2013). The Metabolic Syndrome. In *Genomic and Personalized Medicine* (pp. 1006–1016). Elsevier. https://doi.org/10.1016/B978-0-12-382227-7.00083-5

Lapillonne, A., Clarke, S. D., & Heird, W. C. (2003). Plausible mechanisms for effects of long-chain polyunsaturated fatty acids on growth. *The Journal of Pediatrics*, *143*(4), 9–16. https://doi.org/10.1067/S0022-3476(03)00397-4

Laufer, B. I., Hasegawa, Y., Zhang, Z., Hogrefe, C. E., Del Rosso, L. A., Haapanan, L., Hwang, H., Bauman, M. D., Van de Water, J. A., Taha, A. Y., Slupsky, C. M., Golub, M. S., Capitanio, J. P., VandeVoort, C. A., Walker, C. K., & LaSalle, J. M. (2021). Multi-omic brain and behavioral correlates of cell-free fetal DNA methylation in

macaque maternal obesity models. *BioRxiv*, 2021.08.27.457952. https://doi.org/10.1101/2021.08.27.457952

Leghi, G. E., & Muhlhausler, B. S. (2016). The effect of n-3 LCPUFA supplementation on oxidative stress and inflammation in the placenta and maternal plasma during pregnancy. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *113*, 33–39. https://doi.org/10.1016/j.plefa.2016.08.010

Li, G., Chen, H., Zhang, W., Tong, Q., & Yan, Y. (2018). Effects of maternal omega-3 fatty acids supplementation during pregnancy/lactation on body composition of the offspring: A systematic review and meta-analysis. *Clinical Nutrition*, *37*(5), 1462–1473. https://doi.org/10.1016/j.clnu.2017.08.002

Li, M., Tian, S., Jin, L., Zhou, G., Li, Y., Zhang, Y., Wang, T., Yeung, C. K. L., Chen, L., Ma, J., Zhang, J., Jiang, A., Li, J., Zhou, C., Zhang, J., Liu, Y., Sun, X., Zhao, H., Niu, Z., … Li, R. (2013). Genomic analyses identify distinct patterns of selection in domesticated pigs and Tibetan wild boars. *Nature Genetics*, *45*(12), 1431–1438. https://doi.org/10.1038/ng.2811

Lillehammer, M., Meuwissen, T. H. E., & Sonesson, A. K. (2011). Genomic selection for maternal traits in pigs. *Journal of Animal Science*, *89*(12), 3908–3916. https://doi.org/10.2527/jas.2011-4044

Liu, R., Liu, X., Bai, X., Xiao, C., & Dong, Y. (2020). Different expression of lipid metabolism-related genes in Shandong black cattle and Luxi cattle based on transcriptome analysis. *Scientific Reports*, *10*(1), 21915. https://doi.org/10.1038/s41598-020-79086-4

Liu, Y., Yang, X., Jing, X., He, X., Wang, L., Liu, Y., & Liu, D. (2018). Transcriptomics analysis on excellent meat quality traits of skeletal muscles of the chinese indigenous min pig compared with the large white breed. *International Journal of Molecular Sciences*, *19*(1). https://doi.org/10.3390/ijms19010021

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., & Darnell, J. (2000). *Molecular Cell Biology* (A. Uzman (ed.); 4th editio). Freeman & Co. https://doi.org/10.1016/S1470-8175(01)00023-6

Lone, A. M., & Taskén, K. (2013). Proinflammatory and immunoregulatory roles of eicosanoids in T-cells. *Frontiers in Immunology*, *4*(JUN), 1–15. https://doi.org/10.3389/fimmu.2013.00130

Lopes-Ramos, C. M., Chen, C.-Y., Kuijjer, M. L., Paulson, J. N., Sonawane, A. R., Fagny, M., Platig, J., Glass, K., Quackenbush, J., & DeMeo, D. L. (2020). Sex Differences in Gene Expression and Regulatory Networks across 29 Human Tissues. *Cell Reports*, *31*(12), 107795. https://doi.org/10.1016/j.celrep.2020.107795

Lv, K., Cai, C., Chen, J., Xu, M., Wan, L., Zhou, M., Du, Y., Ma, X., Wu, X., Tang, X., Qiu, H., Wu, D., Han, Y., & Liu, Y. (2021). Prognostic value of lymphoid marker CD7 expression in acute myeloid leukemia patients undergoing allogeneic hematopoietic cell transplantation in first morphological complete remission. *International Journal of Hematology*, *114*(4), 464–471. https://doi.org/10.1007/

s12185-021-03182-y

Maffei, M., Barone, I., Scabia, G., & Santini, F. (2016). The multifaceted haptoglobin in the context of adipose tissue and metabolism. *Endocrine Reviews*, *37*(4), 403–416. https://doi.org/10.1210/er.2016-1009

Maharani, D., Park, H.-B., Lee, J.-B., Yoo, C.-K., Lim, H.-T., Han, S.-H., Lee, S.-S., Ko, M.-S., Cho, I.-C., & Lee, J.-H. (2013). Association of the gene encoding stearoyl-CoA desaturase (SCD) with fatty acid composition in an intercross population between Landrace and Korean native pigs. *Molecular Biology Reports*, *40*(1), 73–80. https://doi.org/10.1007/s11033-012-2014-0

Manaig, Y., Sandrini, S., Panseri, S., Tedeschi, G., Folch, J., Sánchez, A., Savoini, G., & Agazzi, A. (2022). Low n-6/n-3 Gestation and Lactation Diets Influence Early Performance, Muscle and Adipose Polyunsaturated Fatty Acid Content and Deposition, and Relative Abundance of Proteins in Suckling Piglets. *Molecules*, *27*(9), 2925. https://doi.org/10.3390/molecules27092925

Mármol-Sánchez, E., Cirera, S., Zingaretti, L. M., Jacobsen, M. J., Ramayo-Caldas, Y., Jørgensen, C. B., Fredholm, M., Cardoso, T. F., Quintanilla, R., & Amills, M. (2022). Modeling microRNA-driven post-transcriptional regulation using exon–intron split analysis in pigs. *Animal Genetics*, *00*, 1–14. https://doi.org/10.1111/age.13238

Martínez-Montes, Á. M., Fernández, A., Muñoz, M., Noguera, J. L., Folch, J. M., & Fernández, A. I. (2018). Using genome wide association studies to identify common QTL regions in three different genetic backgrounds based on Iberian pig breed. *PLoS ONE*, *13*(3). https://doi.org/10.1371/journal.pone.0190184

Martinsen, K. H., Ødegård, J., Olsen, D., & Meuwissen, T. H. E. (2015). Genetic variation in efficiency to deposit fat and lean meat in Norwegian Landrace and Duroc pigs1. *Journal of Animal Science*, *93*(8), 3794–3800. https://doi.org/10.2527/jas.2015-9174

McArdel, S. L., Terhorst, C., & Sharpe, A. H. (2016). Roles of CD48 in regulating immunity and tolerance. *Clinical Immunology*, *164*, 10–20. https://doi.org/10.1016/j.clim.2016.01.008

McDermaid, A., Monier, B., Zhao, J., Liu, B., & Ma, Q. (2019). Interpretation of differential gene expression results of RNA-seq data: review and integration. *Briefings in Bioinformatics*, *20*(6), 2044–2054. https://doi.org/10.1093/bib/bby067

MEAT ATLAS. (2014). *Global facts and figures about meat*. https://www.boell.de/en/2014/01/13/meat-atlas-2014-global-facts-and-figures-about-meat

Mentzel, C. M. J., Alkan, F., Keinicke, H., Jacobsen, M. J., Gorodkin, J., Fredholm, M., & Cirera, S. (2016). Joint Profiling of miRNAs and mRNAs Reveals miRNA Mediated Gene Regulation in the Göttingen Minipig Obesity Model. *PLOS ONE*, *11*(11), e0167285. https://doi.org/10.1371/journal.pone.0167285

Merks, J. W. M., Mathur, P. K., & Knol, E. F. (2012). New phenotypes for new breeding goals in pigs. *Animal*, *6*(4), 535–543. https://doi.org/10.1017/S1751731111002266

Mitchaothai, J., Yuangklang, C., Wittayakun, S., Vasupen, K., Wongsutthavas, S.,

Srenanul, P., Hovenier, R., Everts, H., & Beynen, A. C. (2007). Effect of dietary fat type on meat quality and fatty acid composition of various tissues in growing–finishing swine. *Meat Science*, *76*(1), 95–101. https://doi.org/10.1016/j.meatsci.2006.10.017

Moore, C. A. C., Milano, S. K., & Benovic, J. L. (2007). Regulation of Receptor Trafficking by GRKs and Arrestins. *Annual Review of Physiology*, *69*(1), 451–482. https://doi.org/10.1146/annurev.physiol.69.022405.154712

Muñoz, M., Bozzi, R., García, F., Núñez, Y., Geraci, C., Crovetti, A., García-Casco, J., Alves, E., Škrlep, M., Charneca, R., Martins, J. M., Quintanilla, R., Tibau, J., Kušec, G., Djurkin-Kušec, I., Mercat, M. J., Riquet, J., Estellé, J., Zimmer, C., … Óvilo, C. (2018). Diversity across major and candidate genes in European local pig breeds. *PLoS ONE*, *13*(11), 1–30. https://doi.org/10.1371/journal.pone.0207475

Naeli, P., Winter, T., Hackett, A. P., Alboushi, L., & Jafarnejad, S. M. (2022). The intricate balance between microRNA-induced mRNA decay and translational repression. *The FEBS Journal*. https://doi.org/10.1111/febs.16422

National Research Council. (2012). Nutrient Requirements of Swine. In *Nutrient Requirements of Swine* (11th ed.). National Academies Press. https://doi.org/10.17226/6016

Nelson, D. L., & Cox, M. M. (2008). *Lehninger principles of biochemistry* (5th editio). W. H. Freeman and Company.

Newcom, D. W., Baas, T. J., & Lampe, J. F. (2002). Prediction of intramuscular fat percentage in live swine using real-time ultrasound1. *Journal of Animal Science*, *80*(12), 3046–3052. https://doi.org/10.2527/2002.80123046x

Nguyen, T. X., Agazzi, A., Comi, M., Bontempo, V., Guido, I., Panseri, S., Sauerwein, H., Eckersall, P. D., Burchmore, R., & Savoini, G. (2020). Effects of low ω6:ω3 ratio in sow Diet and seaweed supplement in piglet diet on performance, colostrum and milk fatty acid profiles, and oxidative status. *Animals*, *10*(11), 2049. https://doi.org/10.3390/ani10112049

Nicolas, C., Lacasa, D., Giudicelli, Y., Demarne, Y., Agli, B., Lecourtier, M.-J., & Lhuillery, C. (1991). Dietary (n-6) Polyunsaturated Fatty Acids Affect β-Adrenergic Receptor Binding and Adenylate Cyclase Activity in Pig Adipocyte Plasma Membrane. *The Journal of Nutrition*, *121*(8), 1179–1186. https://doi.org/10.1093/jn/121.8.1179

Nieto, G., & Ros, G. (2012). Modification of fatty acid composition in meat through diet: effect on lipid peroxidation and relationship to nutritional quality – a review. In A. Catala (Ed.), *Lipid Peroxidation*. InTech. https://doi.org/10.5772/51114

Nold, R. (2006). *Meat quality*. Pork Information Gateway. https://porkgateway.org/wp-content/uploads/2015/07/meat-quality1.pdf

Norwood, J. (2013). *Utilization of fatty acids in swine diets*. Feed Energy Company.

Ntambi, J. M., & Bené, H. (2001). Polyunsaturated Fatty Acid Regulation of Gene

Expression. *Journal of Molecular Neuroscience*, *16*(2–3), 273–278. https://doi.org/10.1385/JMN:16:2-3:273

Núñez-Hernández, F., Pérez, L. J., Muñoz, M., Vera, G., Accensi, F., Sánchez, A., Rodríguez, F., & Núñez, J. I. (2017). Differential expression of porcine microRNAs in African swine fever virus infected pigs: a proof-of-concept study. *Virology Journal*, *14*(1), 198. https://doi.org/10.1186/s12985-017-0864-8

O'Brien, J., Hayder, H., Zayed, Y., & Peng, C. (2018). Overview of microRNA biogenesis, mechanisms of actions, and circulation. *Frontiers in Endocrinology*, *9*(AUG), 1–12. https://doi.org/10.3389/fendo.2018.00402

OECD/FAO. (2018). *OECD-FAO agricultural outlook 2018-2027 - chapter 6: meat*. OECD. https://doi.org/10.1787/agr_outlook-2018-en

Ogawa, M., Kanda, T., Higuchi, T., Takahashi, H., Kaneko, T., Matsumoto, N., Nirei, K., Yamagami, H., Matsuoka, S., Kuroda, K., & Moriyama, M. (2019). Possible association of arrestin domain-containing protein 3 and progression of non-alcoholic fatty liver disease. *International Journal of Medical Sciences*, *16*(7), 909–921. https://doi.org/10.7150/ijms.34245

Olsen, T., Lee, S., Vinknes, K., Norheim, F., Hjorth, M., Turner, C., Refsum, H., Jensen4, J., Birkeland, K., & Drevon, C. (2021). *Tissue Expression of Methyltransferases in Response to Acute and Long-term Exercise in Sedentary Men*. https://doi.org/10.21203/rs.3.rs-143201/v1

Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., & Mann, M. (2006). Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks. *Cell*, *127*(3), 635–648. https://doi.org/10.1016/j.cell.2006.09.026

Paschos, G. K., Ibrahim, S., Song, W.-L., Kunieda, T., Grant, G., Reyes, T. M., Bradfield, C. A., Vaughan, C. H., Eiden, M., Masoodi, M., Griffin, J. L., Wang, F., Lawson, J. A., & FitzGerald, G. A. (2012). Obesity in mice with adipocyte-specific deletion of clock component Arntl. *Nature Medicine*, *18*(12), 1768–1777. https://doi.org/10.1038/nm.2979

Patience, J. F. (2010). Strategies to maximize wean-to-finish net income: Focus on feed and other technical issues. In R. O. Ball (Ed.), *Advances in Pork Production* (pp. 75–85). University of Alberta.

Patwari, P., Emilsson, V., Schadt, E. E., Chutkow, W. A., Lee, S., Marsili, A., Zhang, Y., Dobrin, R., Cohen, D. E., Larsen, P. R., Zavacki, A. M., Fong, L. G., Young, S. G., & Lee, R. T. (2011). The Arrestin Domain-Containing 3 Protein Regulates Body Mass and Energy Expenditure. *Cell Metabolism*, *14*(5), 671–683. https://doi.org/10.1016/j.cmet.2011.08.011

Pelley, J. W. (2011). *Elsevier's Integrated Review Biochemistry E-Book: with STUDENT CONSULT Online Access* (Elsevier Health Sciences (ed.)).

Peng, J., Zhao, J.-S., Shen, Y.-F., Mao, H.-G., & Xu, N.-Y. (2015). MicroRNA Expression Profiling of Lactating Mammary Gland in Divergent Phenotype Swine Breeds. *International Journal of Molecular Sciences*, *16*(1), 1448–1465. https://doi.org/10

.3390/ijms16011448

Pereira, S. L., Leonard, A. E., & Mukerji, P. (2003). Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *68*(2), 97–106. https://doi.org/10.1016/S0952-3278(02)00259-4

Petersen, H. H., Ersbøll, A. K., Jensen, C. S., & Nielsen, J. P. (2002). Serum-haptoglobin concentration in Danish slaughter pigs of different health status. *Preventive Veterinary Medicine*, *54*(4), 325–335. https://doi.org/10.1016/S0167-5877(02)00054-5

Podolska, A., Anthon, C., Bak, M., Tommerup, N., Skovgaard, K., Heegaard, P. M., Gorodkin, J., Cirera, S., & Fredholm, M. (2012). Profiling microRNAs in lung tissue from pigs infected with Actinobacillus pleuropneumoniae. *BMC Genomics*, *13*(1), 459. https://doi.org/10.1186/1471-2164-13-459

Puig-Oliveras, A., Ramayo-Caldas, Y., Corominas, J., Estellé, J., Pérez-Montarelo, D., Hudson, N. J., Casellas, J., Folch, J. M., & Ballester, M. (2014). Differences in Muscle Transcriptome among Pigs Phenotypically Extreme for Fatty Acid Composition. *PLoS ONE*, *9*(6), e99720. https://doi.org/10.1371/journal.pone.0099720

Ratti, M., Lampis, A., Ghidini, M., Salati, M., Mirchev, M. B., Valeri, N., & Hahne, J. C. (2020). MicroRNAs (miRNAs) and Long Non-Coding RNAs (lncRNAs) as New Tools for Cancer Therapy: First Steps from Bench to Bedside. *Targeted Oncology*, *15*(3), 261–278. https://doi.org/10.1007/s11523-020-00717-x

Rauw, W. M., Kanis, E., Noordhuizen-Stassen, E. N., & Grommers, F. J. (1998). Undesirable side effects of selection for high production efficiency in farm animals: A review. *Livestock Production Science*, *56*(1), 15–33. https://doi.org/10.1016/S0301-6226(98)00147-X

Realini, C. E., Duran-Montgé, P., Lizardo, R., Gispert, M., Oliver, M. A., & Esteve-Garcia, E. (2010). Effect of source of dietary fat on pig performance, carcass characteristics and carcass fat content, distribution and fatty acid composition. *Meat Science*, *85*(4), 606–612. https://doi.org/10.1016/j.meatsci.2010.03.011

Ritchie, M. D., Holzinger, E. R., Li, R., Pendergrass, S. A., & Kim, D. (2015). Methods of integrating data to uncover genotype-phenotype interactions. *Nature Reviews Genetics*, *16*(2), 85–97. https://doi.org/10.1038/nrg3868

Romero, R., Espinoza, J., Gotsch, F., Kusanovic, J. P., Friel, L. A., Erez, O., Mazaki-Tovi, S., Than, N. G., Hassan, S., & Tromp, G. (2006). The use of high-dimensional biology (genomics, transcriptomics, proteomics, and metabolomics) to understand the preterm parturition syndrome. *BJOG: An International Journal of Obstetrics and Gynaecology*, *113*(SUPPL. 3), 118–135. https://doi.org/10.1111/j.1471-0528.2006.01150.x

Rooke, J. ., SinclaiR, A. G., & Ewen, M. (2001a). Changes in piglet tissue composition at birth in response to increasing maternal intake of long-chain n -3 polyunsaturated fatty acids are non-linear . *British Journal of Nutrition*, *86*(4), 461–470. https://doi.org/10

.1079/bjn2001422

Rooke, J. ., SinclaiR, A. G., & Ewen, M. (2001b). Changes in piglet tissue composition at birth in response to increasing maternal intake of long-chain n -3 polyunsaturated fatty acids are non-linear. *British Journal of Nutrition*, *86*(4), 461–470. https://doi.org/10 .1079/BJN2001422

Ros-Freixedes, R., Gol, S., Pena, R. N., Tor, M., Ibáñez-Escriche, N., Dekkers, J. C. M., & Estany, J. (2016). Genome-Wide Association Study Singles Out SCD and LEPR as the Two Main Loci Influencing Intramuscular Fat Content and Fatty Acid Composition in Duroc Pigs. *PLOS ONE*, *11*(3), e0152496. https://doi.org/10.1371/ journal.pone.0152496

Rosenvold, K., & Andersen, H. J. (2003). Factors of significance for pork quality - A review. *Meat Science*, *64*(3), 219–237. https://doi.org/10.1016/S0309-1740(02)00186 -9

Rovadoscki, G. A., Pertile, S. F. N., Alvarenga, A. B., Cesar, A. S. M., Pértille, F., Petrini, J., Franzo, V., Soares, W. V. B., Morota, G., Spangler, M. L., Pinto, L. F. B., Carvalho, G. G. P., Lanna, D. P. D., Coutinho, L. L., & Mourão, G. B. (2018). Estimates of genomic heritability and genome-wide association study for fatty acids profile in Santa Inês sheep. *BMC Genomics*, *19*(1), 375. https://doi.org/10.1186/s12864-018-4777-8

Sainz, R. D., & Hasting, E. (2000). Simulation of the development of adipose tissue in beef cattle. In *Modelling nutrient utilization in farm animals.* (pp. 175–182). CABI. https://doi.org/10.1079/9780851994499.0175

Sakayori, N., Katakura, M., Hamazaki, K., Higuchi, O., Fujii, K., Fukabori, R., Iguchi, Y., Setogawa, S., Takao, K., Miyazawa, T., Arita, M., & Kobayashi, K. (2020). Maternal dietary imbalance between omega-6 and omega-3 fatty acids triggers the offspring's overeating in mice. *Communications Biology*, *3*(1), 473. https://doi.org/10.1038/s42003-020-01209-4

Saponaro, C., Gaggini, M., Carli, F., & Gastaldelli, A. (2015). The Subtle Balance between Lipolysis and Lipogenesis: A Critical Point in Metabolic Homeostasis. *Nutrients*, *7*(11), 9453–9474. https://doi.org/10.3390/nu7115475

Sarkar, S., Dey, B. K., & Dutta, A. (2010). MiR-322/424 and -503 are induced during muscle differentiation and promote cell cycle quiescence and differentiation by down-regulation of Cdc25A. *Molecular Biology of the Cell*, *21*(13), 2138–2149. https://doi.org/10.1091/mbc.e10-01-0062

Sauerwein, H., Schmitz, S., & Hiss, S. (2005). The acute phase protein haptoglobin and its relation to oxidative status in piglets undergoing weaning-induced stress. *Redox Report*, *10*(6), 295–302. https://doi.org/10.1179/135100005X83725

Scheenstra, M. R., van Harten, R. M., Veldhuizen, E. J. A., Haagsman, H. P., & Coorens, M. (2020). Cathelicidins modulate TLR-activation and inflammation. *Frontiers in Immunology*, *11*(June), 1–16. https://doi.org/10.3389/fimmu.2020.01137

Schmitz, G., & Ecker, J. (2008). The opposing effects of n-3 and n-6 fatty acids. *Progress*

in Lipid Research, *47*(2), 147–155. https://doi.org/10.1016/j.plipres.2007.12.004

Schook, L. B., Beever, J. E., Rogers, J., Humphray, S., Archibald, A., Chardon, P., Milan, D., Rohrer, G., & Eversole, K. (2005). Swine Genome Sequencing Consortium (SGSC): A Strategic Roadmap for Sequencing The Pig Genome. *Comparative and Functional Genomics*, *6*(4), 251–255. https://doi.org/10.1002/cfg.479

Segura, J., Calvo, L., Óvilo, C., González-Bulnes, A., Olivares, A., Cambero, M. I., & López-Bote, C. J. (2015). Alternative method for intramuscular fat analysis using common laboratory equipment. *Meat Science*, *103*, 24–27. https://doi.org/10.1016/j.meatsci.2014.12.011

Sessler, A. M., & Ntambi, J. M. (1998). Polyunsaturated Fatty Acid Regulation of Gene Expression. *The Journal of Nutrition*, *128*(6), 923–926. https://doi.org/10.1093/jn/128.6.923

Shimano, H., & Sato, R. (2017). SREBP-regulated lipid metabolism: convergent physiology — divergent pathophysiology. *Nature Reviews Endocrinology*, *13*(12), 710–730. https://doi.org/10.1038/nrendo.2017.91

Siwan, E., Twigg, S. M., & Min, D. (2022). Alterations of CD163 expression in the complications of diabetes: A systematic review. *Journal of Diabetes and Its Complications*, *36*(4), 108150. https://doi.org/10.1016/j.jdiacomp.2022.108150

Sosnicki, A. A., Matthews, N., Fields, B., & Jungst, S. (2010). *Pork fat quality*. Cutting Edge. https://en.engormix.com/pig-industry/articles/pork-fat-quality-t34693.htm

Soudais, C., de Villartay, J.-P., Le Deist, F., Fischer, A., & Lisowska-Grospierre, B. (1993). Independent mutations of the human CD3–ε gene resulting in a T cell receptor/CD3 complex immunodeficiency. *Nature Genetics*, *3*(1), 77–81. https://doi.org/10.1038/ng0193-77

Stryer, L., Berg, J. M., & Tymoczko, J. L. (2002). *Biochemistry* (5th editio). W. H. Freeman and Company.

Sun, R., Yang, Y., Gu, Z., Tang, X., Zhang, C., Kou, W., & Wei, P. (2019). Silencing of CD86 in dendritic cells by small interfering RNA regulates cytokine production in T cells from patients with allergic rhinitis in-vitro. *Molecular Medicine Reports*. https://doi.org/10.3892/mmr.2019.10638

Szostak, A., Ogłuszka, M., te Pas, M. F. W., Poławska, E., Urbański, P., Juszczuk-Kubiak, E., Blicharski, T., Pareek, C. S., Dunkelberger, J. R., Horbańczuk, J. O., & Pierzchała, M. (2016). Effect of a diet enriched with omega-6 and omega-3 fatty acids on the pig liver transcriptome. *Genes & Nutrition*, *11*(1), 9. https://doi.org/10.1186/s12263-016-0517-4

Szűcs, I., & Vida, V. (2017). Global tendencies in pork meat - production, trade and consumption. *Applied Studies in Agribusiness and Commerce*, *11*(3–4), 105–111. https://doi.org/10.19041/APSTRACT/2017/3-4/15

Tanghe, S., & De Smet, S. (2013). Does sow reproduction and piglet performance benefit from the addition of n-3 polyunsaturated fatty acids to the maternal diet? *The*

Veterinary Journal, *197*(3), 560–569. https://doi.org/10.1016/j.tvjl.2013.03.051

Thornton, P. K. (2010). Livestock production: recent trends, future prospects. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1554), 2853–2867. https://doi.org/10.1098/rstb.2010.0134

Vamathevan, J. J., Hall, M. D., Hasan, S., Woollard, P. M., Xu, M., Yang, Y., Li, X., Wang, X., Kenny, S., Brown, J. R., Huxley-Jones, J., Lyon, J., Haselden, J., Min, J., & Sanseau, P. (2013). Minipig and beagle animal model genomes aid species selection in pharmaceutical discovery and development. *Toxicology and Applied Pharmacology*, *270*(2), 149–157. https://doi.org/10.1016/j.taap.2013.04.007

van der Steen, H. A. M., Prall, G. F. W., & Plastow, G. S. (2005). Application of genomics to the pork industry. *Journal of Animal Science*, *83*(suppl_13), E1–E8. https://doi.org/https://doi.org/10.2527/2005.8313_supplE1x

Villé, H., Rombouts, G., Van Hecke, P., Perremans, S., Maes, G., Spincemaille, G., & Geers, R. (1997). An evaluation of ultrasound and nuclear magnetic resonance spectroscopy to measure in vivo intramuscular fat content of longissimus muscle of pigs. *Journal of Animal Science*, *75*(11), 2942. https://doi.org/10.2527/1997.75112942x

Virk, R., Buddenbaum, N., Al-Shaer, A., Armstrong, M., Manke, J., Reisdorph, N., Sergin, S., Fenton, J. I., Wallace, E. D., Ehrmann, B. M., Lovins, H. B., Gowdy, K. M., Smith, M. R., Smith, G. J., Kelada, S. N. P., & Shaikh, S. R. (2022). Obesity reprograms the pulmonary polyunsaturated fatty acid-derived lipidome, transcriptome, and gene-oxylipin networks. *Journal of Lipid Research*, *63*(10), 100267. https://doi.org/10.1016/j.jlr.2022.100267

Wada, Y., Yoshida-Yamamoto, S., Wada, Y., Nakayama, M., Mitsuda, N., & Kitajima, H. (2017). Trans fatty acid accumulation in the human placenta. *Journal of Mass Spectrometry*, *52*(3), 139–143. https://doi.org/10.1002/jms.3910

Wang, C., Arrington, J., Ratliff, A. C., Chen, J., Horton, H. E., Nie, Y., Yue, F., Hrycyna, C. A., Tao, W. A., & Kuang, S. (2019). Methyltransferase-like 21c methylates and stabilizes the heat shock protein Hspa8 in type I myofibers in mice. *Journal of Biological Chemistry*, *294*(37), 13718–13728. https://doi.org/10.1074/jbc.RA119.008430

Wang, H. L., Zhu, Z. M., Wang, H., Yang, S. L., Zhao, S. H., & Li, K. (2006). Molecular characterization and association analysis of porcine CA3. *Cytogenetic and Genome Research*, *115*(2), 129–133. https://doi.org/10.1159/000095232

Wang, J., Hua, L., Chen, J., Zhang, J., Bai, X., Gao, B., Li, C., Shi, Z., Sheng, W., Gao, Y., & Xing, B. (2017). Identification and characterization of long non-coding RNAs in subcutaneous adipose tissue from castrated and intact full-sib pair Huainan male pigs. *BMC Genomics*, *18*(1), 542. https://doi.org/10.1186/s12864-017-3907-z

Wang, K., Huang, R., Tong, X., Zhang, K., Liu, Y., Zeng, F., Hu, H., & Jiang, T. (2019). Molecular and clinical characterization of TMEM71 expression at the transcriptional level in glioma. *CNS Neuroscience & Therapeutics*, *25*(9), 965–975. https://doi.org/

10.1111/cns.13137

Wang, W., Zhu, J., Lyu, F., Panigrahy, D., Ferrara, K. W., Hammock, B., & Zhang, G. (2014). ω-3 Polyunsaturated fatty acids-derived lipid metabolites on angiogenesis, inflammation and cancer. *Prostaglandins & Other Lipid Mediators*, *113–115*, 13–20. https://doi.org/10.1016/j.prostaglandins.2014.07.002

Wang, Z., Shang, P., Li, Q., Wang, L., Chamba, Y., Zhang, B., Zhang, H., & Wu, C. (2017). iTRAQ-based proteomic analysis reveals key proteins affecting muscle growth and lipid deposition in pigs. *Scientific Reports*, *7*(1), 46717. https://doi.org/10.1038/srep46717

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics*, *10*(1), 57–63. https://doi.org/10.1038/nrg2484

Ward, S. L. (2014). *Omics, bioinformatics, computational biology*. AltTox.Org. http://alttox.org/mapp/emerging-technologies/omics-bioinformatics-computational-biology/

Webster, J. (2012). *Animal Husbandry Regained* (1st ed.). Routledge. https://doi.org/10.4324/9780203094228

Wei, Z., & Wong, G. W. (2013). Roles of Micro-RNAs in Metabolism. In *Encyclopedia of Biological Chemistry: Second Edition* (2nd ed.). Elsevier Inc. https://doi.org/10.1016/B978-0-12-378630-2.00078-5

Weinberg, Z. Y., & Puthenveedu, M. A. (2019). Regulation of G protein-coupled receptor signaling by plasma membrane organization and endocytosis. *Traffic*, *20*(2), 121–129. https://doi.org/10.1111/tra.12628

Wiederstein, J. L., Nolte, H., Günther, S., Piller, T., Baraldo, M., Kostin, S., Bloch, W., Schindler, N., Sandri, M., Blaauw, B., Braun, T., Hölper, S., & Krüger, M. (2018). Skeletal Muscle-Specific Methyltransferase METTL21C Trimethylates p97 and Regulates Autophagy-Associated Protein Breakdown. *Cell Reports*, *23*(5), 1342–1356. https://doi.org/10.1016/j.celrep.2018.03.136

Wilund, K. R. (2007). Is the anti-inflammatory effect of regular exercise responsible for reduced cardiovascular disease? *Clinical Science*, *112*(11), 543–555. https://doi.org/10.1042/CS20060368

Woodward, A. M., Lehoux, S., Mantelli, F., Di Zazzo, A., Brockhausen, I., Bonini, S., & Argüeso, P. (2019). Inflammatory Stress Causes N-Glycan Processing Deficiency in Ocular Autoimmune Disease. *The American Journal of Pathology*, *189*(2), 283–294. https://doi.org/10.1016/j.ajpath.2018.10.012

Xu, J., Xu, Y., Li, Y., Jadhav, K., You, M., Yin, L., & Zhang, Y. (2016). Carboxylesterase-1 is regulated by hepatocyte nuclear factor 4α and protects against alcohol- and MCD diet-induced liver injury. *Scientific Reports*, *6*(1), 24277. https://doi.org/10.1038/srep24277

Yang, X., Li, L., Chai, X., & Liu, J. (2022). The association between ST8SIA2 gene and

behavioral phenotypes in children with autism spectrum disorder. *Frontiers in Behavioral Neuroscience*, *16*. https://doi.org/10.3389/fnbeh.2022.929878

Yao, H.-T., Chang, Y.-W., Lan, S.-J., Chen, C.-T., Hsu, J. T. A., & Yeh, T.-K. (2006). The inhibitory effect of polyunsaturated fatty acids on human CYP enzymes. *Life Sciences*, *79*(26), 2432–2440. https://doi.org/10.1016/j.lfs.2006.08.016

Ye, L., Su, X., Wu, Z., Zheng, X., Wang, J., Zi, C., Zhu, G., Wu, S., & Bao, W. (2012). Analysis of Differential miRNA Expression in the Duodenum of Escherichia coli F18-Sensitive and -Resistant Weaned Piglets. *PLoS ONE*, *7*(8), e43741. https://doi.org/10.1371/journal.pone.0043741

Yonekura, S., & Ueda, K. (2021). EVI2B Is a New Prognostic Biomarker in Metastatic Melanoma with IFNgamma Associated Immune Infiltration. *Cancers*, *13*(16), 4110. https://doi.org/10.3390/cancers13164110

Yu, K., Shu, G., Yuan, F., Zhu, X., Gao, P., Wang, S., Wang, L., Xi, Q., Zhang, S., Zhang, Y., Li, Y., Wu, T., Yuan, L., & Jiang, Q. (2013). Fatty acid and transcriptome profiling of longissimus dorsi muscles between pig breeds differing in meat quality. *International Journal of Biological Sciences*, *9*(1), 108–118. https://doi.org/10.7150/ijbs.5306

Zalewska, M., Siara, M., & Sajewicz, W. (n.d.). G protein-coupled receptors: abnormalities in signal transmission, disease states and pharmacotherapy. *Acta Poloniae Pharmaceutica*, *71*(2), 229–243.

Zaragosi, L.-E., Wdziekonski, B., Brigand, K. Le, Villageois, P., Mari, B., Waldmann, R., Dani, C., & Barbry, P. (2011). Small RNA sequencing reveals miR-642a-3p as a novel adipocyte-specific microRNA and miR-30 as a key regulator of human adipogenesis. *Genome Biology*, *12*(7), R64. https://doi.org/10.1186/GB-2011-12-7-R64

Zeibig, S., Büttcher, M., Goebel, S., Pauli, J., Hunger, A., Ungerer, M., Gawaz, M., & Münch, G. (2019). The Scavenger Receptor CD68 Regulates Platelet Mediated Oxidized Low-Density Lipoprotein (oxLDL) Deposition in Atherosclerotic Vessels at an Early Stage of Atherosclerosis in LDLR-/-/ApoBec-/- Mice. *Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology*, *52*(4), 681–695. https://doi.org/10.33594/000000048

Zhang, H., Li, M., Kaboli, P. J., Ji, H., Du, F., Wu, X., Zhao, Y., Shen, J., Wan, L., Yi, T., Wen, Q., Li, X., Cho, C. H., Li, J., & Xiao, Z. (2021). Identification of cluster of differentiation molecule-associated microRNAs as potential therapeutic targets for gastrointestinal cancer immunotherapy. *The International Journal of Biological Markers*, *36*(2), 22–32. https://doi.org/10.1177/17246008211005473

Zhao, Y., Rao, J., Qiu, T., Li, C., & Zhou, X. (2021). The Effect of RBP4 on microRNA Expression Profiles in Porcine Granulosa Cells. *Animals*, *11*(5), 1391. https://doi.org/10.3390/ani11051391

Zhong, W., Jiang, Z., Zheng, C., Lin, Y., Yang, L., & Zou, S. (2011). Relationship between proteome changes of Longissimus muscle and intramuscular fat content in finishing

pigs fed conjugated linoleic acid. *British Journal of Nutrition*, *105*(1), 1–9. https://doi.org/10.1017/S0007114510003181

Zivkovic, A. M., Telis, N., German, J. B., & Hammock, B. D. (2011). Dietary omega-3 fatty acids aid in the modulation of inflammation and metabolic health. *California Agriculture*, *65*(3), 106–111. https://doi.org/10.3733/ca.v065n03p106

Zjablovskaja, P., Kardosova, M., Danek, P., Angelisova, P., Benoukraf, T., Wurm, A. A., Kalina, T., Sian, S., Balastik, M., Delwel, R., Brdicka, T., Tenen, D. G., Behre, G., Fiore, F., Malissen, B., Horejsi, V., & Alberich-Jorda, M. (2017). EVI2B is a C/EBPα target gene required for granulocytic differentiation and functionality of hematopoietic progenitors. *Cell Death & Differentiation*, *24*(4), 705–716. https://doi.org/10.1038/cdd.2017.6

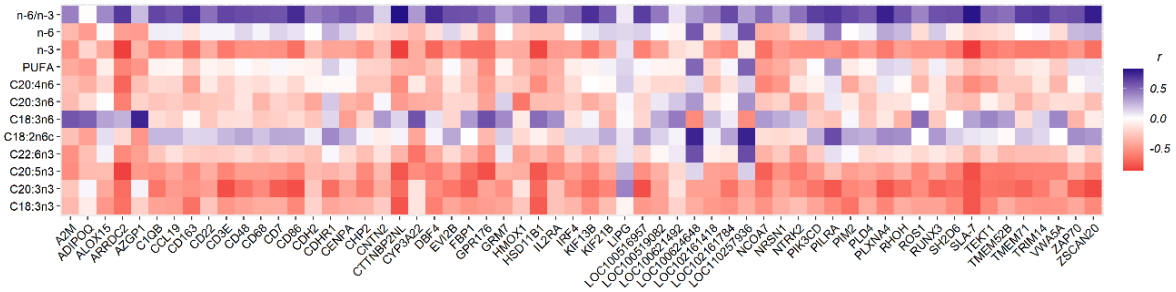
ANNEXES

Chapter 7

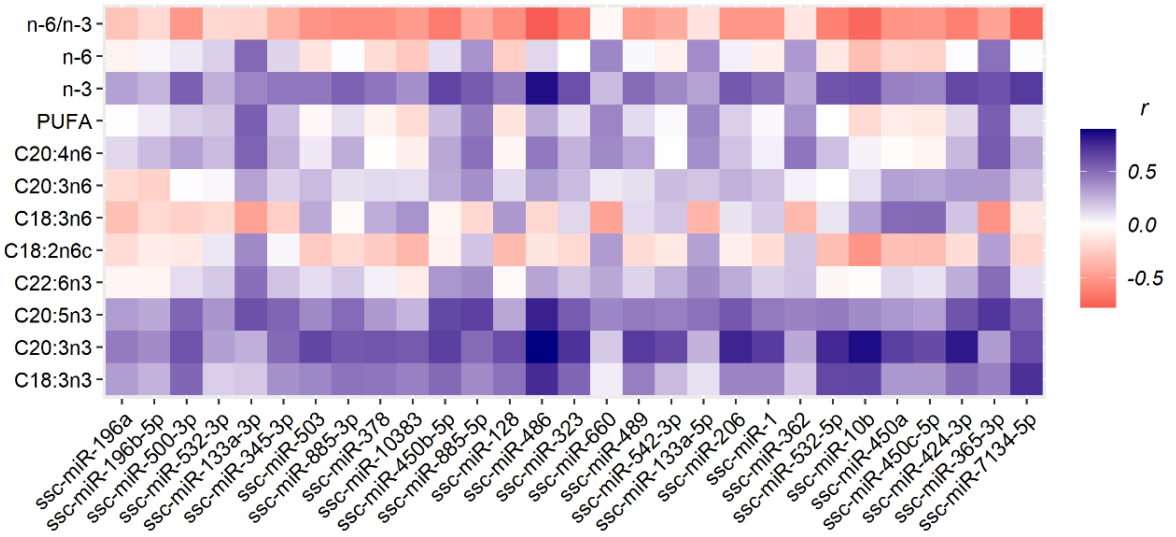
7.1 Supplementary Materials Paper II: All Supplementary Tables, Figures and related Documents included and referred in the published paper (Paper II) that form part of the present Ph.D. thesis are available at its corresponding online version:

https://www.mdpi.com/article/10.3390/molecules27092925/s1

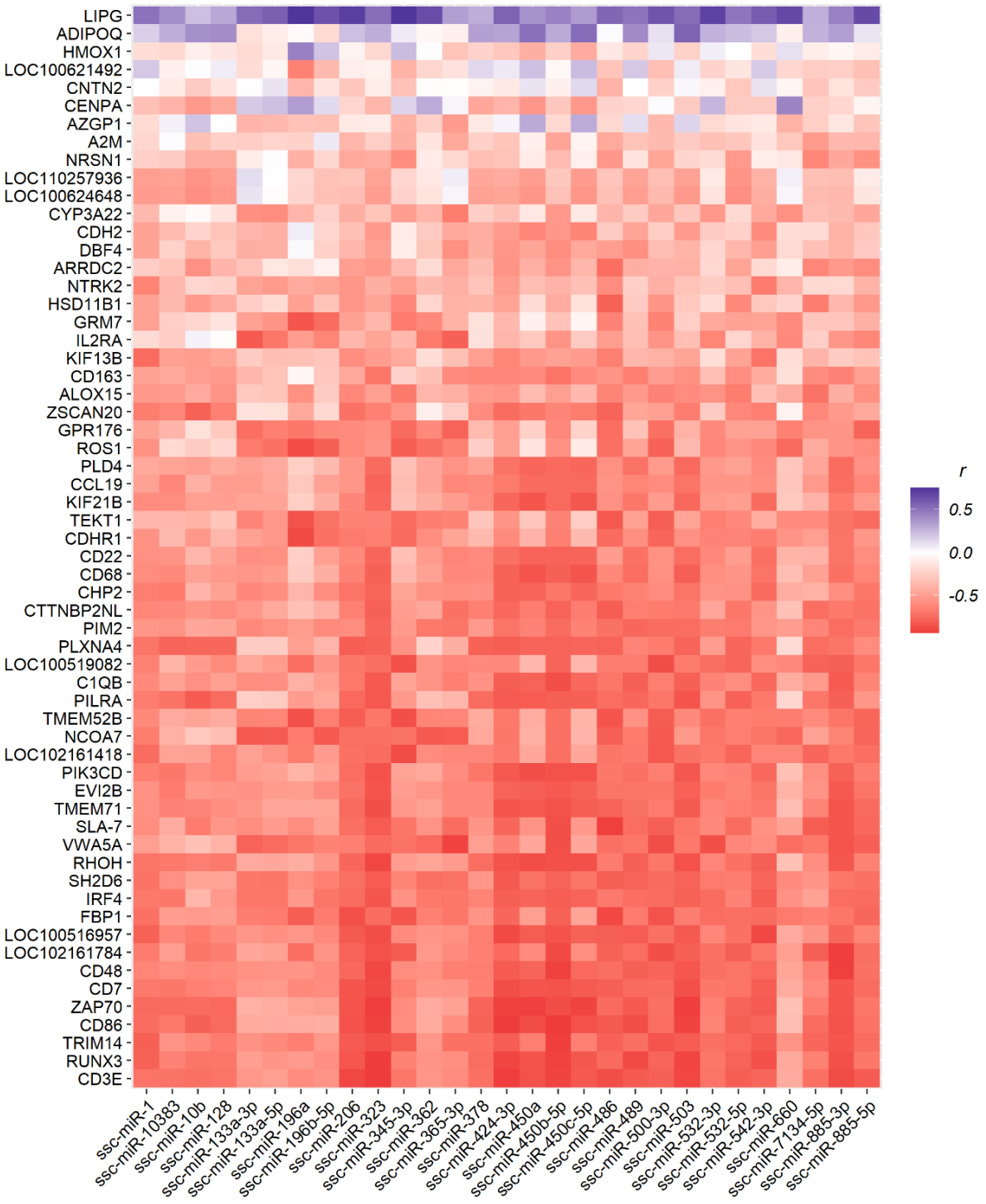
7.2. Supplementary Figures Paper III: "Exon-intron split analysis reveals post-transcriptional regulatory signals induced by high and low n-6/n-3 polyunsaturated fatty acid ratio diets in piglets"



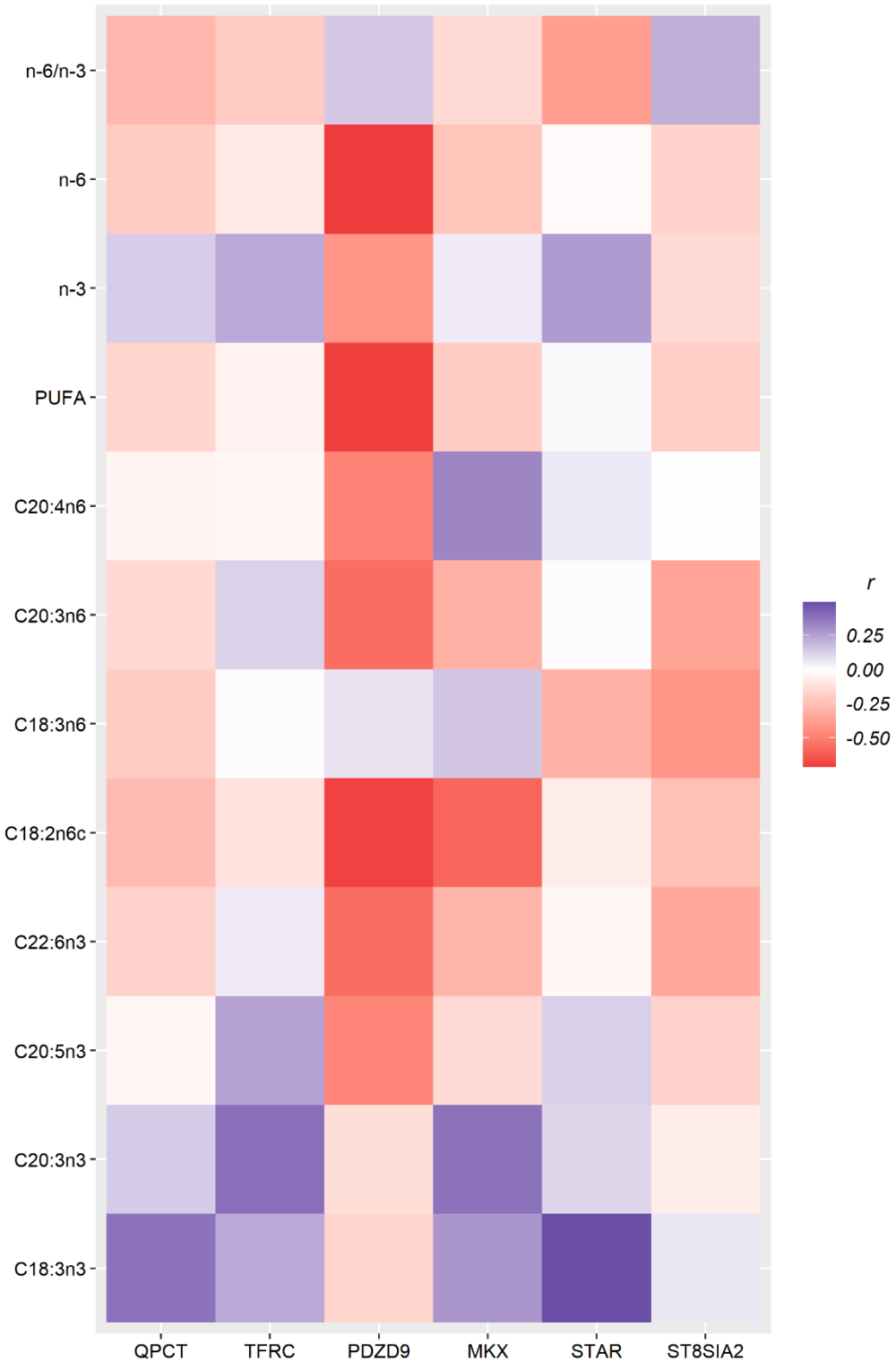
Paper III: Additional Figure 1a. Heatmap plot showing correlation values among mRNA genes with the top 5% negative PTc scores and at least 1.5-fold reduction in ΔEx values and PUFA profiles of *longissimus dorsi* skeletal muscle in **SOY-M** vs **LIN-M** piglets.



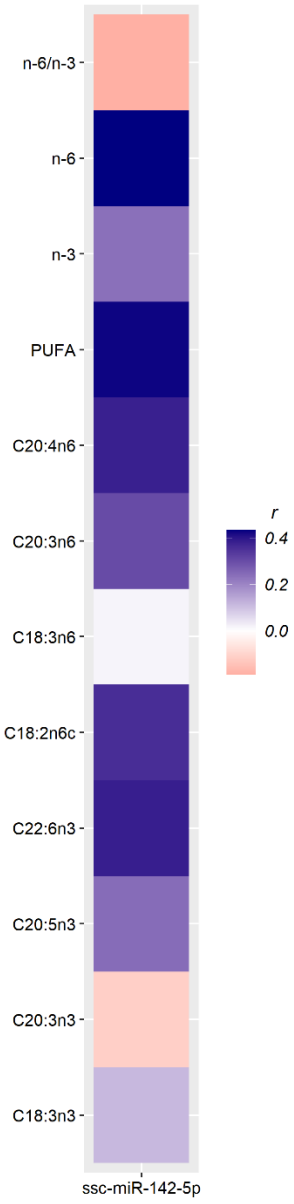
Paper III: Additional Figure 1b. Heatmap plot showing correlation values among upregulated miRNAs and PUFA profiles of *longissimus dorsi* skeletal muscle in SOY-M vs LIN-M piglets.



Paper III: Additional Figure 1c. Heatmap plot showing correlation values among mRNA genes with the top 5% negative PTc scores and at least 1.5-fold reduction in ΔEx values and upregulated miRNAs in **SOY-M** vs **LIN-M** piglets.



Paper III: Additional Figure 2a. Heatmap plot showing correlation values among mRNA genes with the top 5% negative PTc scores and at least 1.5-fold reduction in ΔEx values and PUFA profiles of *longissimus dorsi* skeletal muscle in **SOY-F** vs **LIN-F** piglets.



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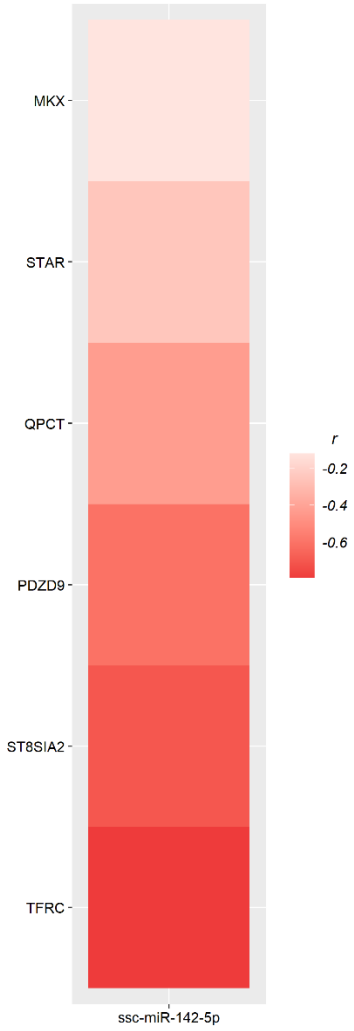
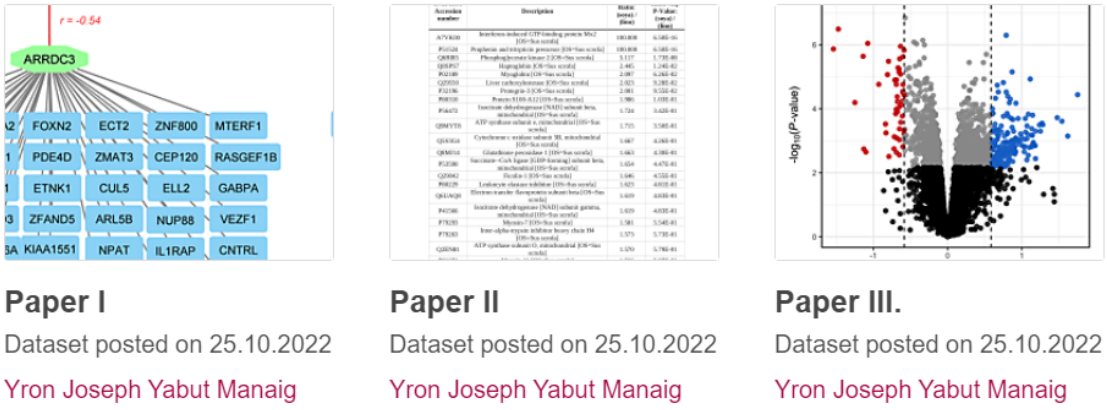


(2c)

Paper III: Additional Figure 2b. Heatmap plot showing correlation values among upregulated miRNAs and PUFA profiles of *longissimus dorsi* skeletal muscle in **SOY-F** vs **LIN-F** piglets; **Additional Figure 2c.** Heatmap plot showing correlation values among mRNA genes with the top 5% negative PTc scores and at least 1.5-fold reduction in ΔEx values and upregulated miRNAs in **SOY-F** vs **LIN-F** piglets.

7.3. Supplementary Materiels on Figshare. Additionally, all Supplementary Tables, Figures and related Documents for Paper I, Paper II, and Paper III are publicly available and can be downloaded from the following link:

https://figshare.com/projects/PhD_Thesis_Annexes_Omics_Approaches_For_n-6n-3_Polyunsaturated_Fatty_Acid_Ratio_In_Pigs_Yron_Joseph_Yabut_Manaig/151644



Annex Figure 1. Folders on Figshare, an online open access repository, where all supplementary materials of Papers I, II, and III are uploaded.

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Chapter 8

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To **Ate Kuy**, my Ate Kuy, you are the epitome of a Warrior. Thank you for showering us with your endless love, guidance, and care since we were kids. Thank you for giving me a home away from home from the other side of the world. I know that life has not been really easy but you continue to demonstrate that faith and perseverance can do wonders and miracles. No words will ever capture how much we love and appreciate you. *¡Te quiero mucho Ate Kuy y mil abrazos y besitos para ti!*

And above everything else, to my **Guide** up there, who has always been my strength, my savior, my rock, and my stronghold, for the endless guidance, wisdom, blessings, love and mercy, and for delivering these aforementioned people that helped me to reach this feat. I did all of these because of You. To Thee, I lift up everything.