

Accepted Manuscript

Analysis of meat quality traits and gene expression profiling of pigs divergent in residual feed intake

Justyna Horodyska, Michael Oster, Henry Reyer, Anne Maria Mullen, Peadar G. Lawlor, Klaus Wimmers, Ruth M. Hamill



PII: S0309-1740(17)30848-3
DOI: doi:[10.1016/j.meatsci.2017.11.021](https://doi.org/10.1016/j.meatsci.2017.11.021)
Reference: MESC 7416
To appear in: *Meat Science*
Received date: 2 June 2017
Revised date: 8 September 2017
Accepted date: 16 November 2017

Please cite this article as: Justyna Horodyska, Michael Oster, Henry Reyer, Anne Maria Mullen, Peadar G. Lawlor, Klaus Wimmers, Ruth M. Hamill , Analysis of meat quality traits and gene expression profiling of pigs divergent in residual feed intake. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. *Mesc*(2017), doi:[10.1016/j.meatsci.2017.11.021](https://doi.org/10.1016/j.meatsci.2017.11.021)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Analysis of meat quality traits and gene expression profiling of pigs divergent in residual feed intake

Justyna Horodyska^{1,2}, Michael Oster², Henry Reyer², Anne Maria Mullen¹, Peadar G. Lawlor³, Klaus Wimmers^{2,4}, Ruth M. Hamill^{1*}

¹ *Teagasc, Food Research Centre, Ashtown, Dublin 15, Ireland*

² *Leibniz Institute for Farm Animal Biology (FBN), Institute for Genome Biology, Dummerstorf, Germany*

³ *Teagasc, Pig Development Department, AGRIC, Moorepark, Fermoy, Co. Cork, Ireland*

⁴ *Faculty of Agricultural and Environmental Sciences, University Rostock, Rostock, Germany*

**Corresponding author*

Email addresses

J. Horodyska: Justyna.Horodyska@teagasc.ie

M. Oster: oster@fhn-dummerstorf.de

H. Reyer: reyer@fhn-dummerstorf.de

A.M. Mullen: anne.mullen@teagasc.ie

P.G. Lawlor: peadar.lawlor@teagasc.ie

K. Wimmers: wimmers@fhn-dummerstorf.de

R.M. Hamill*: Ruth.Hamill@teagasc.ie Tel. +353 1 8059933

Abstract

Residual feed intake (RFI), the difference between actual feed intake and predicted feed requirements, is suggested to impact various aspects of meat quality. The objective of this study was to investigate the molecular mechanisms underpinning the relationship between RFI and meat quality. Technological, sensory and nutritional analysis as well as transcriptome profiling were carried out in *Longissimus thoracis et lumborum* muscle of pigs divergent in RFI (n=20). Significant differences in sensory profile and texture suggest a minor impairment of meat quality in more efficient pigs. Low RFI animals had leaner carcasses, greater muscle content and altered fatty acid profiles compared to high RFI animals. Accordingly, differentially expressed genes were enriched in muscle growth and lipid & connective tissue metabolism. Differences in protein synthesis and degradation suggest a greater turnover of low RFI muscle, while divergence in connective tissue adhesion may impact tenderness. Fatty acid oxidation tending towards suppression could possibly contribute to reduced mitochondrial activity in low RFI muscle.

Keywords: pork quality, feed efficiency, FE, RFI, transcriptomics, RNA

Abbreviations

RFI residual feed intake

LTL *Longissimus thoracis et lumborum*

IMF Intramuscular fat content

Pm post-mortem

LRFI low RFI

HRFI high RFI

WBSF Warner Bratzler shear force

mRNA messenger RNA

miRNA microRNA

1. Introduction

Producing meat more feed efficiently has been a major goal in pig breeding. Improvement in pig production efficiency is being targeted through selection for enhanced feed efficiency (FE), which is an indicator of an animal's efficiency in converting feed into live weight (Wilkinson, 2011). Residual feed intake (RFI), a measure of FE, refers to a difference between an individual's actual feed intake and its expected nutritional requirements due to maintenance and growth (Saintilan, et al., 2013). A low RFI indicates a decreased energy requirement for maintenance (Hoque & Suzuki, 2009), therefore less feed is needed and production is more efficient. RFI is a moderately heritable trait with estimates ranging from 0.15 to 0.40, which makes it a suitable trait for genetic improvement (Fan, et al., 2010). A gene expression profiling of skeletal muscle from RFI divergent Large White pigs, carried out using microarrays, revealed 1,000 differentially expressed (DE) probes. Genes significantly up-regulated in low RFI pigs were mainly associated with protein synthesis, while down-regulated genes were predominantly involved in mitochondrial energy metabolism (Vincent, et al., 2015). Moreover, RNA sequencing of skeletal muscle in RFI divergent Yorkshire boars identified 99 DE genes. Similarly to the study carried out on Large White pigs described above, genes associated with skeletal muscle differentiation and proliferation were up-regulated and genes involved in mitochondrial energy metabolism were down-regulated in low RFI pigs (Jing, et al., 2015). Although a high level of correlation between microarray and RNA sequencing platforms have been reported, shorter and less abundant transcripts have a higher possibility to be detected using microarray approach (Nazarov, et al., 2017). Therefore in these cases and particularly in quantifying microRNA, microarray might outperform RNA sequencing (Git, et al., 2010; Nazarov, et al., 2017). Nevertheless, one of the downsides of using microarrays is that a number of genes / mRNA isoforms are not incorporated (Bumgarner, 2013). There is evidence that selection for reduced RFI pigs is associated with leaner carcasses with greater muscle content and reduced back-fat thickness (Lefaucheur, et al., 2011). Moreover, decreased intramuscular fat content and ultimate pH as well as greater drip loss and lighter meat colour have been reported in meat produced from low RFI pigs (Faure, et al., 2013). Nevertheless, the relationship between RFI and meat quality is not fully elucidated and the biological processes associated with RFI which impact meat quality are not well understood. Therefore the objectives of this study were to investigate 1) the technological, sensory and nutritional quality of pork from pigs

differing in RFI, 2) the molecular mechanisms induced in muscle tissue contributing to differences in RFI using microarray platform and 3) the functional networks underpinning the potential relationship between meat quality and RFI.

2. Materials and Methods

2.1. Animals and experimental design

Animal care, slaughter and tissue collection of the animals used in this study were performed in compliance with national regulations related to animal research and commercial slaughtering and were approved by the local committees for the care and use of animals of the Teagasc and the Leibniz Institute for Farm Animal Biology. This study involved 80 pigs (39 gilts and 41 boars) from the cross Maxgro (Hermitage Genetics) x (German Landrace x Large White), which represented the intact litters of 7 sows inseminated with semen from 4 boars (Hermitage Genetics, Kilkenny, Ireland) having a high estimated breeding value for feed conversion efficiency. As previously described in Metzler-Zebell, et al. (2017), pigs were weaned at 28 days of age and sibling groups were group-housed. All pigs were provided with the same sequence of diets, with the same ingredient and chemical composition (starter, link, weaner and finisher), via Feed Intake Recording Equipment (FIRE) feeders (Schauer Agrotonic, Wels, Austria). Pigs had *ad libitum* access to feed and water. Pigs were placed on test between day 42 and 91 post-weaning, whereby intake was recorded daily, and pig weight and back-fat depth was recorded weekly. Average daily feed intake (ADFI) and average daily gain (ADG) from day 42 to day 91 post-weaning were calculated for each pig. RFI was calculated at the end of the test period as the residuals from a least squares regression model of ADFI on ADG, metabolic live weight, gender and also all relevant two-way interactions, and the effects of back-fat and muscle depth using the PROC REG procedure in SAS (version 9.4; SAS Inst. Inc., Cary, NC, USA). Pigs were categorised as the highest and lowest RFI within litter and gender. Out of the 80 pigs, a total of 20 divergent sib-pairs of gilts (10 low (L) and 10 high (H) RFI from lowest and highest quartile, respectively) with an average final body weight of 101kg (SD: 7.6kg) (average birth weight: 1.2kg, SD: 0.2kg) were selected for transcriptomic and meat quality evaluation. Growth performance parameters of the pigs selected for further evaluation are depicted in Table S1. Prior to slaughter, the animals were fasted for 18 hours. The pigs were electronically stunned followed by exsanguination. A sample of the *Longissimus thoracis et lumborum* (LTL) muscle was collected from each carcass, cut up finely and preserved in RNALater[®] (Ambion Inc., Austin, USA) within 10

minutes post-slaughter. It was then stored overnight at 4°C followed by storage at -80°C until RNA isolation. For meat quality measurements, the LTL muscle was excised from each carcass 24 hours *post-mortem* (*pm*).

2.2. Meat and carcass quality measurements

Carcass grading. Based on the difference in light reflectance of tissues, carcass was measured for fat thickness and muscle depth between 3rd and 4th rib on the day of slaughter using Hennessy Grading Probe (Hennessy Grading Systems Ltd., Auckland, New Zealand).

pH. pH of LTL muscle was measured at 45 minutes (pH 45m), 3 hours (pH 3h) and 24 hours (pH 24h) *pm*, using a portable Hanna pH meter (Hanna Instruments, Woonsocket, RI, USA). A previously calibrated pH probe was inserted in the LTL between the 12th and 13th rib.

Colour. Meat colour of the fresh chops and after 1h of blooming was recorded at day 1 *pm*. It was measured with MiniScan XE Plus (Hunter Associates Laboratory Inc., Virginia, USA) using CIE L* (lightness), a* (redness) and b* (yellowness) colour scale. The measurements were taken at three locations on each chop and averaged.

Drip loss (DL). Drip loss was measured using the bag method of Honikel (1998), whereby pork chops measuring 2.5cm in thickness were trimmed of the adipose tissue and epimysium at day 1 *pm*, to a weight of 80g±1g. Each pork chop was then suspended by string inside an inflated plastic bag, ensuring that the meat did not make direct contact with the bag. The bag was then sealed and suspended for 48 h at 4 °C. Thereafter, the samples were dried with paper towel, weighed and drip loss was expressed as percentage of the original weight of the chop.

Cook loss (CL). Samples frozen at -20°C on day 1 and day 7 *pm* were thawed in bags in a circulating water bath at 20°C. The muscle chops were then trimmed of external fat to a similar size, dried with a paper towel and weighed. They were placed in plastic bags and immersed in a water bath (Grant Instruments Ltd., England) at 77°C until they reached a core temperature of 75°C was measured with a temperature probe (Eirelec Ltd., Ireland). The samples were allowed to cool at room temperature. Weight of the chops was recorded followed by refrigerated storage. The cook loss was expressed as a percentage of the raw weight of the chop.

Warner Bratzler shear force (WBSF). After cook loss was determined, the samples were used to measure WBSF according to AMSA guidelines, 1995. Briefly, six cores of 1.25cm diameter were obtained from each sample. The cores were cut in parallel to the longitudinal orientation of fibres and were sheared perpendicularly to the muscle fibres long axis with a shear blade using 500N load cell at a crosshead speed of 50mm/min (Instron model 5543). Data was analysed using Blue Hill software (Instron Ltd., Buckinghamshire, UK).

Protein, moisture

and intramuscular fat (IMF) & mineral content. Samples frozen at -20°C on day 1 *pm* were thawed in bags in a circulating water bath at 20°C . Muscle chops were then trimmed of external fat and homogenized using a Robot Coupe blender (R301 Ultra, Robot coupe SA, France). Protein content was measured with a Leco Nitrogen/Protein Analyser (FP-528, Leco Corp., MI, USA) using the Dumas method in accordance with AOAC method 992.15, 1990. IMF and moisture were measured with NMR Smart Trac & Smart 5 Rapid Fat and Moisture Analyser (CEM Corporation, USA) using AOAC method 985.14 and 985.26, 1990. Ash content was determined by calcination of the meat samples in a muffle furnace at 540°C (AOAC method 923.03). The analysis for each sample was carried out in duplicate and the mean recorded. **Fatty acid (FA) profile.** Frozen samples (day 1 *pm*) were transported to a commercial laboratory for FA profile analysis. Intramuscular fat was extracted from LTL muscle and fatty acid methyl esters were prepared and analysed using Gas Chromatography - Flame Ionization Detector (GC - FID) in accordance with SAL Cam Nut003 method (Pearson's Chemical Analysis of Foods, 9th Edition, Longman Group UK Limited, 1991, 0-582-40910-1). Results were expressed as mg fatty acid per 100g meat. **Sensory assessment.** Panellists received a total of 60 hours training in red meat texture, flavour and after-effects (AMSA, 2015). Prior to assessment of the actual samples, the trained panellists were familiarised with pork samples from the sample set. Accuracy and repeatability of the panellists were then examined using PanelCheck software (version 2, 1991, Free Software Foundation, Inc., MA, USA). Vacuum packed chops were aged at 4°C for 7 days followed by freezing. A day before sensory assessment, the chops were thawed overnight in the fridge. The chops were then grilled (Velox grill, Silesia Velox UK Ltd., Oxfordshire, England) until the core temperature reached 70°C and cut into 2.5 cm x 2 cm cubes. The 20 samples were combined with an additional set of 40 samples from a separate trial. A total of 60 samples were assessed over 5 sessions. Each 3-hour session consisted of 12 randomised samples and was evaluated by trained panellists ($n=8$) rating the pork chops sensory attributes on a scale of 1 (very poorly detectable attribute) to 100 (extremely detectable attribute). **Statistical analysis.** PROC MIXED procedure in the SAS system was used to evaluate associations between RFI and meat quality traits in the Maxgro x (Landrace x Large White) gilts ($n = 20$). The model included RFI groups as a fixed effect, sow as a random effect and pre-slaughter live weight as a covariate, as well as the absolute values of RFI as a weight statement to account for differences in RFI values within the RFI groups.

2.3. RNA isolation and cDNA synthesis

According to the manufacturer's protocol total RNA from preserved LTL muscle of 10 biological replicates per RFI group was extracted using Tri-Reagent (Sigma-Aldrich, Taufkirchen, Germany). It was then subjected to DNase treatment and a column-based purification using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). RNA samples were analysed for integrity and quantity using agarose gel electrophoresis and Nanodrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). RNA samples were also checked for DNA contamination by PCR of the porcine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (Forward primer: AAGCAGGGATGATGTTCTGG; Reverse primer: ATGCCTCCTGTACCACCAAC). For cDNA synthesis, 1 µg of total RNA was utilized in presence of random primers (Promega, Mannheim, Germany), oligo (dT) primer and Superscript[®] III reverse transcriptase (Invitrogen Corp., San Diego, CA, USA).

2.4. Microarray hybridisation

Double stranded cDNA was synthesised using total RNA, which served as a template in the subsequent *in vitro* transcription reaction. Antisense cRNA was then purified and sense-strand cDNA was synthesised by the reverse transcription of cRNA, using a GeneChip[®] WT Amplification Kit (Affymetrix, Santa Clara, CA, USA). Biotin-labeled cDNA was then fragmented with GeneChip[®] WT Terminal Labeling Kit (Affymetrix) and injected onto porcine *snowball* arrays (Affymetrix) containing 47,845 probe sets with a mean coverage of 22 probes per transcript (Freeman, et al., 2012). The arrays were incubated for 16 hours at 45°C in Affymetrix GeneChip Hybridization Oven 640. After hybridisation, the arrays were washed and stained with streptavidin-phycoerythrin antibody solution (Affymetrix) on an Affymetrix GeneChip Fluidic Station 450 station. The arrays were then scanned with Affymetrix GeneChip Scanner 3000. Microarray images were quantified using GCOS 1.1.1 (Affymetrix) and raw data was deposited in a MIAME-compliant database, the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) (accession number: GSE99653).

2.5. Analysis of microarray data & functional annotation

Bioinformatic analysis of the microarrays, including pre-processing and normalisation, was implemented using R packages (affy, arrayQualityMetrics, genefilter, affyPLM and vsn) (version 3.1.1, <https://www.R-project.org/>). Robust multi-array average (RMA) normalisation (Log₂) was performed and probe sets with a low standard deviation ($\text{std} \leq 0.23$) were discarded. A further filtering step involved filtering by both control probe sets and means (means ≤ 2.5 were rejected). PROC MIXED including RFI groups and sow as fixed effects

and birth weight as a covariate was implemented in JMP Genomics 6 software of SAS (version 6, SAS INST.) to determine relative changes in transcript abundances (fold change). The corresponding false discovery rate (q-value) (Storey & Tibshirani, 2003) was calculated using the qvalue R package (version 3.1.1, <https://www.R-project.org/>). Relative changes in transcript abundance significant at the nominal 0.05 level were subjected to further ontology analysis (Ingenuity Pathway Analysis; Ingenuity[®] Systems, <http://www.ingenuity.com/>). Benjamini-Hochberg corrected p-values were used to detect the most significant canonical pathways ($P < 0.05$) and to map the genes to the most significant molecular and cellular functions ($P < 0.05$) contained in the IPA library. Functional annotations with a z-score > 2 were considered significantly activated in low RFI pigs. Information contained in the Ingenuity[®] Knowledge Base was used to create potential important networks of DE genes. A list of DE microRNAs (miRNAs; represented on the *snowball* array) along with DE transcripts and related fold changes ($P \leq 0.05$) were submitted to Ingenuity miRNA Target Filter (<http://www.ingenuity.com/>) to predict target transcripts regulated by these miRNAs and to investigate miRNA–mRNA expression patterns. Additionally, potential networks of the DE transcripts that were predicted to be regulated by the DE miRNAs were generated.

2.6. Microarray validation

In order to validate the microarray results, six DE genes involved in lipid and energy metabolism (*ACACA*, *ACSL1*, *BCL2*, *MMP2*, *SDHB* and *TFAM*) were selected for quantitative real-time PCR (qPCR), whereby *RPL10* and *RPL32* were used as housekeeping genes. Primers for target genes were designed online with Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>) based on *Sus scrofa* nucleotide sequences. Specificity of primers was determined with the BLAST search tool in the NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST>). Primer sets for selected reference and target genes are listed in Table S2. qPCR was carried out on 96-well plates using 7500 system (Applied Biosystems, Foster City, CA, USA). 2 μ l of cDNA was amplified in a 21 μ l reaction volume using 10 μ l Power SYBR[®] Green Master Mix, 0.15 μ l (10 μ M) of each forward and reverse primer and 8.7 μ l nuclease free water (QIAGEN Ltd., West Sussex, UK). All qPCR reactions were performed in triplicate for each cDNA sample. Cycling conditions for reference and DE genes of interest were as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. In order to confirm a specificity of all individual amplification reactions, a dissociation curve analysis was included at the end of the amplification: 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s. A standard curve using 5-fold serial

dilutions of a cDNA pool was generated to which relative expression values were compared. A normalization factor obtained from the expression of the reference genes was utilised for normalization of the candidate DE genes' expression values. Data was analysed using a general linear model procedure in the SAS system (version 9.3, SAS INST.) Spearman correlations between the microarray expression values (\log_2) and qPCR normalised relative expression values (Ct) were carried out using R (version 3.1.1, <https://www.R-project.org/>).

3. Results

3.1. Meat quality

The mean RFI (g/day) of the L (low RFI - LRFI) and H group (high RFI - HRFI) was -106.6 (SD: 78.9) and 86.4 (SD: 84.7), respectively. Carcass and technological meat quality traits of LTL muscle in LRFI and HRFI pigs are shown in Table 1 and sensory attributes of LTL muscle in LRFI and HRFI pigs are presented in Fig 2. Muscle depth and percent lean meat significantly differed between the RFI groups ($P < 0.05$), with the LRFI having greater lean meat percentage and increased muscle depth. LRFI pigs were also found to have significantly reduced IMF percentage ($P < 0.05$). The pH at 45m *pm* had a tendency toward increased values in the LRFI group ($P = 0.055$), however pH at 3h and 24h *pm* did not differ between the two groups. Moreover meat from LRFI pigs tended to have increased cook loss at day 1 *pm* ($P = 0.053$) but there was no difference detected at day 7 *pm*. Muscle from LRFI pigs was significantly associated with increased WBSF (less tender) at day 1 *pm* ($P < 0.01$) and had a tendency towards increased WBSF scores at day 7 *pm* ($P = 0.057$). Significant difference in tenderness between the RFI groups was also detected by sensory panellists who scored the LRFI meat (day 7 *pm*) as less tender ($P < 0.05$). Furthermore, meat from LRFI pigs was found to have lower b^* values ($P < 0.05$) compared to meat from HRFI pigs, however the difference in yellowness scores was not significant after 1h blooming ($P > 0.1$). Lightness and redness did not differ between the two groups. Pork sensory assessment also revealed that meat produced from LRFI pigs had higher scores for stringy/fibrous/sinewy and chewy texture ($P < 0.05$). Additionally, meat from LRFI pigs was found less crumbly in texture ($P < 0.05$), less sweet ($P < 0.001$) and more sour ($P < 0.05$). Nutritional composition with regards to fatty acid (FA) content in LTL muscle (mg FA/100g meat), and percentage of FA in IMF of pigs divergent in RFI are presented in Table 2. From a nutritional point of view, meat from LRFI group contained significantly lower amounts of saturated fatty acids (SFA) such as myristic, palmitic and stearic ($P < 0.05$) and monounsaturated fatty acids (MUFA) including palmitoleic, oleic ($P < 0.05$) and eicosenoic ($P < 0.1$). Neither member of polyunsaturated

fatty acid (PUFA) family differed significantly in LTL muscle of RFI divergent pigs. The IMF from LRFI group had significantly higher proportions of PUFA such as linoleic ($P < 0.001$) and alpha-linolenic ($P < 0.05$), and lower level of palmitic acid ($P < 0.01$) belonging to a SFA family. MUFA did not differ significantly in IMF between the RFI groups however.

3.2. Differential mRNA and miRNA expression profile

A total of 30,992 probe-sets remained after filtering and 1,035 probes were differentially expressed between LRFI and HRFI groups (645 probes were up-regulated and 390 were down-regulated in LRFI pigs) ($P \leq 0.05$, $q < 0.75$). Of the 1,035 probes, 875 were annotated and assigned to 800 genes (481 genes were up-regulated and 319 genes were down-regulated in LRFI compared to HRFI pigs) and 33 miRNAs (27 miRNAs were up-regulated and 6 miRNAs were down-regulated in LRFI compared to HRFI pigs). 123 annotated genes and 10 annotated miRNAs were found to be at least 1.5-fold differentially expressed. The most altered genes were *AP2M1* (2.37; LRFI > HRFI) and *NCOA2* (-3.32; LRFI < HRFI), and the most altered miRNAs were mir-675 (1.70 LRFI > HRFI) and mir-4311 (-1.90 LRFI < HRFI). Due to the relatively small differences in gene expression between the low and high RFI groups, transcripts and miRNAs with a $P \leq 0.05$ were considered significantly differentially expressed. To further refine this data, a gene ontology approach was utilised to extract molecular themes and networks.

3.3. Functional annotation, canonical pathway and network analysis

Twenty six molecular and cellular functions were significantly associated with genes DE in relation to RFI ($P < 0.05$). The six most significant biological processes altered in RFI groups were 'gene expression' (150 DE genes), 'lipid metabolism' (82 DE genes), 'molecular transport' (156 DE genes), 'small molecule biochemistry' (113 DE genes), 'cellular growth and proliferation' (248 DE genes), and 'cell death and survival' (227 DE genes). Ten canonical signalling pathways ($P < 0.05$) were significantly over-represented among DE genes in relation to RFI. The six most significant signalling pathways were 'TR/RXR activation' (16 DE genes), 'PEDF signalling' (11 DE genes), 'HIF α signalling' (11 DE genes), 'myc mediated apoptosis signalling' (8 DE genes), 'aryl hydrocarbon receptor signalling' (13 DE genes) and 'adipogenesis pathway' (12 DE genes) (Table 3). Twenty five over-represented gene networks were generated for differentially expressed genes in relation to RFI. The most significant network (Fig 3) was represented by functions related to

‘molecular transport’, ‘nucleic acid metabolism’ and ‘small molecule biochemistry’, and contained 32 differentially expressed *TP53*-associated molecules.

3.4. miRNA-mRNA paired expression profiling and network analysis of target transcripts

Since miRNAs repress gene translation through base pairing with their target mRNAs, identification of their target transcripts is vital for understanding their biological function (Rajewsky, 2006; Tang, et al., 2015). In this study, potential DE target genes regulated by the 33 DE miRNAs were predicted. Of these, 29 miRNAs were mapped and predicted to regulate 379 DE genes. miRNA-mRNA expression pairing was performed to investigate expression patterns of miRNAs and potential mRNA targets. Although some studies reported miRNAs capability to activate gene expression (Orang, Safaralizadeh & Kazemzadeh-Bavili, 2014), our aim was to focus on the miRNA repression of target genes, therefore miRNA-mRNA expression patterns exhibiting the same direction were removed. 168 DE genes targeted by 28 DE miRNAs remained (Table S5), with mir3184, mir4313 and mir631 being the top three miRNAs predicted regulating 79, 44 and 44 DE target transcripts, respectively. In order to gain insights into biological functions of DE miRNAs, molecular connectivity of DE genes regulated by these miRNAs was performed. The most significant network (Fig S1) was represented by functions related to ‘cellular development, growth & proliferation’ and ‘respiratory system development and function’ and contained 24 DE molecules. Another interesting network (Fig 4) included ‘embryonic development’, ‘connective tissue development & function’ and ‘organ morphology’ and was represented by 10 DE molecules.

3.5. Microarray validation

The qPCR confirmed significant differences in the expression of *ACSL1*, *SDHB* and *TFAM* transcripts between LRFI and HRFI groups. mRNA abundances of *ACACA*, *BCL2* and *MMP2* showed a numerical change in the same direction when compared to results obtained from the microarrays. Spearman correlations between microarray expression values and qPCR Ct values were significant ($P < 0.05$) for all transcripts and ranged from 0.56 to 0.84 (Table 4).

4. Discussion

4.1. Meat quality

The first objective of this study was to examine the effect of divergence in RFI on technological, sensory and nutritional quality of pork. Our findings indicate that low RFI pigs

exhibit increased muscle size, leanness and reduced IMF content, and this is consistent with previous studies (Faure, et al., 2013; Lefaucheur, et al., 2011; Smith, et al., 2011). The similar trend in adiposity and marbling is not surprising and can be explained by a positive genetic correlation between the two traits (Lefaucheur, et al., 2011). In agreement with the fact that lipid content can influence colour measurements (Schwab, Baas, Stalder & Mabry, 2006), meat from LRFI pigs was found to be less yellow. The results regarding pH are in contrast with previous studies reporting no effect on early pH and significantly reduced ultimate pH in LRFI pigs, compared to HRFI (Faure, et al., 2013; Lefaucheur, et al., 2011). *Longissimus* (LM) is a large fast-twitch glycolytic muscle composed of approximately 74 percent type IIBW (white) fibres (Lefaucheur, et al., 2011). In a previous study, LM from pigs divergent in RFI showed no difference in proportions of muscle fibre types (Smith, et al., 2011). On the contrary, another study reported higher proportions of IIBW fibres in meat from LRFI pigs compared to HRFI group (Lefaucheur, et al., 2011). Increase in IIBW fibres and consequently greater muscle glycogen content in LRFI pigs have been associated with impaired meat quality through reduced ultimate pH, greater lightness and drip loss (Gilbert, et al., 2007; Lefaucheur, et al., 2011). In the present study, these associations were not observed, which may indicate that muscle from the LRFI pigs does not contain higher proportions of IIBW fibres. Indeed, the array contained several myosin heavy chain isoforms but no significant differences in their expression were observed here between RFI groups. Previous studies, such as that of Lefaucheur et al. (2011) and Faure et al. (2013) utilised a different experimental model for RFI, with animals being selected in divergent lines for multiple generations. The animals in the present study were selected from within families and the mechanisms underpinning the differences in RFI which we observed are likely to differ from those in previous experiments.

Shear force and sensory analysis showed that meat from LRFI pigs was tougher and more stringy compared to HRFI. No significant association with tenderness was found in a previous study carried out on low RFI pigs versus control pigs (Faure, et al., 2013). In contrast, another study (Smith, et al., 2011) did report a significant correlation between tenderness and RFI, and they postulated that tenderness of meat produced by LRFI pigs could be negatively affected by greater calpastatin activity resulting in decreased *post-mortem* protein degradation. They also suggested that the lower IMF in this group of pigs may contribute to decreased tenderness of resultant meat from LRFI pigs. While the relationship between IMF and tenderness is inconclusive (Smith, et al., 2011), many authors have

speculated on a link between IMF and tenderness, due to greater ease of tissue disruption in areas richer in fat cells. Furthermore, trained panellists scored meat from LRFI pigs as less sweet and more sour compared to meat from HRFI group. A previous study also reported that meat with elevated ultimate pH (pH=6) was perceived as more sweet, tender and less acidic than meat with normal pH (Bryhni, et al., 2003). It is interesting that meat from HRFI pigs showed a similar trend in the sensory scores while having an ultimate pH within the normal range and although HRFI pigs had slightly higher values than meat from LRFI pigs the difference was not significant. This variation in sweet and sour taste could also be caused by a diverse proportion of particular amino acids. It has been well documented that variable concentrations of amino acids affect taste perception (Choudhuri, Delay & Delay, 2015). For example L-serine and D-serine amino acids are associated with sweet taste (Kawai, Sekine-Hayakawa, Okiyama & Ninomiya, 2012), whereas glutamic and aspartic amino acids are associated with sour taste (Birch & Kemp, 1989).

Fat content and fatty acid composition play important roles in sensory attributes and nutritional value of meat (Nieto & Ros, 2012). Meat contains considerable concentrations of cell membrane phospholipids, which are rich in PUFA (Wood, et al., 2008). Nevertheless the most predominant class of fatty acids present in meat are MUFA followed by SFA, which are the major constituents of triacylglycerol (Kasprzyk, Tyra & Babicz, 2015; Wood, et al., 2008). Reduced IMF content is frequently associated with higher proportion of PUFA and decreased levels of SFA and MUFA (Dominguez & Lorenzo, 2014; Dugan, et al., 2015; Wood, et al., 2008). Here, low RFI muscle was associated with reduced IMF content and, in keeping with previous studies, this was predominantly due to reduced SFA and MUFA content, with similar total meat PUFA content in LTL muscle of both groups. The health profile of muscle from LRFI pigs could be considered more beneficial, being 42 percent lower in SFA and with IMF 19 percent richer in PUFA.

4.2. Gene expression profile

Besides the carcass and meat quality analysis of the RFI divergent pigs, the other aim of this study was to attain insights on the biological events explaining differences in RFI and increase understanding of the biological processes linking RFI and meat quality traits such as sensory profile and technological performance. The magnitude of DE genes found in the current study is similar to a recently performed gene expression profiling of skeletal muscle of Large White pigs selected for divergent RFI (Vincent, et al., 2015). It is important to note

that the experimental model used in our study involved pairs of animals from the lowest and highest quartile chosen within the same family, i.e. divergent sib-pairs, and the work conducted by Vincent et al. (2015) utilised animals of divergently selected lines for RFI over several generations. As a result of using animals from the same family, the genetic variability, and transcriptomic response is likely due to different mendelian inheritance of parental alleles within the sib-pairs that largely contribute to the trait of interest, rather than long term selection effects and genetic drift. Hence it is of interest that very similar numbers of differentially expressed transcripts were observed in both experiments. Furthermore, downstream validation of the microarray results via qPCR of selected DE genes was successful thus confirming reliability of the expression profiles. Functional annotation revealed a number of biological processes related to growth, connective tissue and lipid metabolism suggesting that these might be important mechanisms contributing to differences in RFI.

4.2.1. Growth

Adaptor related protein complex 2 mu 1 subunit (*AP2M1*) was the most up-regulated gene (fold change = 2.37) in LRFI pigs. *AP2M1* is a subunit of adaptor protein 2 (*AP2*) involved in clathrin-mediated endocytosis, which is the uptake of nutrients from the surface of the cell into the cell via clathrin-coated vesicles (Tian, Chang, Fan, Flajolet & Greengard, 2013). Accordingly, functional annotation revealed endocytosis significantly activated in LRFI pigs (z-score = 3.11). Nuclear receptor coactivator 2 (*NCOA2*) was the most down-regulated gene (fold change = -3.32) in LRFI pigs. *NCOA2* belongs to the nuclear receptor coactivator family, which assists in the function of nuclear hormone receptors playing a vital role in cell growth, development and homeostasis via regulating expression of particular genes. In a previous study, porcine *NCOA2* transcript was positively associated with IMF content in LM muscle (Wang, et al., 2008). This is in agreement with the LRFI pigs exhibiting suppressed *NCOA2* expression and reduced IMF content. Two miRNAs, mir135a and mir3184, that were predicted to suppress *NCOA2* expression were significantly over-expressed in LRFI pigs. Thus these miRNAs could play a mechanistic role in reducing IMF content through inhibiting *NCOA2* activity in LRFI pigs.

In agreement with increased muscle depth and decreased IMF content in LRFI pigs, functional annotation revealed a number of biological processes related to growth. Specifically, 'cell survival' and 'cell differentiation' were significantly activated (z-score =

2.3 and 2.4 respectively) in LRFI pigs. Amongst the up-regulated genes were insulin like growth factor 2 (*IGF2*). Protein encoded by *IGF2* plays an essential role in skeletal muscle differentiation (Alzhanov, McInerney & Rotwein, 2010) and lean versus intramuscular fat content (Aslan, et al., 2012; Van Laere, et al., 2003). Other significantly enriched functions related to growth were 'protein synthesis and degradation', with meltrin-alpha (*ADAM12*) being over-expressed in LRFI pigs. *ADAM12* is an adhesion molecule that has previously been found to play a role in skeletal muscle development and regeneration (Kurisaki, et al., 2003; Moghadaszadeh, et al., 2003; Przewozniak, et al., 2013). Up-regulation of *ADAM12* along with other genes involved in muscle protein synthesis, and also up-regulation of muscle protein degrading enzymes such as calpain 10 (*CAPN10*) and caspase 9 (*CASP9*) suggest greater muscle protein turnover in LRFI pigs. This can be supported by another study reporting up-regulation of genes involved in processes related to protein synthesis and degradation, more specifically 'ribonucleoprotein complex biogenesis' and 'ubiquitin-dependent catabolic process' in LRFI pigs (Gondret, et al., 2017). Vincent et al. (2015) also identified greater muscle protein synthesis in LRFI pigs and also a calpain, in that case calpain 2 to be over-expressed in muscle from LRFI pigs. While probes corresponding to known players in *pm* muscle proteolysis such as calpain 1 and calpastatin, (Geesink, Taylor & Koohmaraie, 2005) were not incorporated in the array, *CASP9* which is an initiator of *pm* proteolysis that has been suggested to influence meat tenderness thus the rate of proteolysis (Ouali, et al., 2006) was up-regulated in LRFI pigs. Other studies reported either no difference (Le Naou, Le Floch, Louveau, Gilbert & Gondret, 2012) or reduced protein degradation in LRFI pigs (Cruzen, et al., 2013; Smith, et al., 2011). Lobley (2003) postulated that muscle growth driven by enhanced synthesis and decreased degradation of protein contributes to net gain in deposition. Nevertheless, protein synthesis is much more energetically costly in comparison to protein degradation (Lobley, 2003). Our data supports the possibility that LRFI pigs reuse existing proteins and thus conserve energy, which otherwise would be utilised for protein synthesis, directing it towards more efficient muscle growth. In addition, the interactions depicted in the tumor suppressor p53 (*TP53*) rooted network (Figure 3) support the role of *TP53* as a central hub in mediating the modulation of muscle cell growth and differentiation (Porrello, et al., 2000; Tamir & Bengal, 1998). *FXDY* domain containing ion transport regulator 3 (*FXDY3*) and chloride intracellular channel 2 (*CLIC2*), coding for integral membrane proteins that regulate function of ion channels (Biasiotta, D'Arcangelo, Passarelli, Nicodemi & Facchiano, 2016; Littler, et al., 2010), were differentially expressed in this network. Ion channels are vital modulators of apoptosis

through permitting release of potassium and chloride ions subsequently leading to cell shrinkage (Wanitchakool, et al., 2016). TP53 regulated inhibitor of apoptosis 1 (*TRIAPI1*) and ADP ribosylation factor like GTPase interacting protein 1 (*ARL6IP1*) were another over-represented genes in this network. Proteins encoded by these genes are involved in apoptosis via regulating *CASP9* activity (Adams, et al., 2015; Lui, Chen, Wang & Naumovski, 2003). The connection of these DE genes to *TP53*, which was over-expressed in LRFI pigs, suggests their importance in lean growth due to its involvement in cell proliferation. Moreover, nineteen miRNAs were predicted to be associated with ‘cellular development, growth & proliferation’ through regulation of their target DE genes.

4.2.2 Connective tissue

A number of DE genes were significantly overrepresented in ‘adhesion of connective tissue’ function with a tendency towards activation in the high FE pigs (z-score = 1.99). During the process of muscle growth, connective tissue undergoes dynamic remodelling which involves its proteolytic degradation, and the establishment of expanded networks through synthesis of new connective tissue components (Purslow, 2014). Accordingly, LRFI pigs which showed signs of increased muscle mass also over-expressed matrix metalloproteinase 2 (*MMP2*) belonging to a family of enzymes associated with connective tissue degradation and remodelling (Rodier, El Moudni, Kauffmann-Lacroix, Daniault & Jacquemin, 1999; Woessner, 1991). This pattern might be expected to be associated with more tender meat, which is often associated with elevated turnover of muscle proteins (Olsson & Pickova, 2005) but this was not observed in the present study. Collagen type I alpha 1 chain (*COL1A1*), one of the predominant collagen types in the skeletal muscle (McCormick, 1994), was up-regulated in LRFI pigs. Over-expression of *COL1A1* was previously associated with increased drip loss (McBryan, Hamill, Davey, Lawlor & Mullen, 2010; Ponsuksili, et al., 2008). In the present study, while drip loss did not differ significantly between RFI groups, a tendency towards increased cook loss at day 1 *pm* was observed in meat from LRFI pigs. Upon heating, collagen fibrils shrink which consequently leads to fluid loss (Weston, Rogers & Althen, 2002). This finding may relate to the stringy/fibrous/sinewy and chewy texture of meat produced by LRFI pigs. Eleven miRNAs were predicted to regulate expression of genes with functions related to ‘connective tissue development & function’. Down-regulation of mir584 and mir887 potentially resulted in the over-expression of collagen type I alpha 2 chain (*COL1A2*) and connective tissue growth factor (*CTGF*), respectively.

4.2.3. Lipid and energy metabolism

In accordance with the relation of RFI to IMF content, a number of DE genes were significantly enriched in ‘adipogenesis pathway’ and lipid metabolism functions. Peroxisome proliferator activated receptor gamma (*PPARG*) is a master regulator of adipogenesis in a variety of tissues (Norris, et al., 2003). Overexpression of *PPARG* in adipocytes of LRFI pigs would suggest enhanced adipogenesis which disagrees with the observed shifts in muscle to fat ratio. However, considering that skeletal muscle is a highly heterogeneous tissue, the observed differences in *PPARG* expression could be attributable either to muscle cells or intramuscular adipocytes. A previous study reported that muscle-specific deletion of *PPARG* is associated with enhanced adiposity in mice (Norris, et al., 2003), which could explain the muscle of LRFI pigs exhibiting reduced IMF content while enhanced *PPARG* expression. Acyl-CoA synthetase long-chain family member 1 (*ACSL1*) expression was suppressed in LRFI pigs. The enzyme encoded by this gene is involved in lipid synthesis through conversion of fatty acids to triglycerides (Parkes, et al., 2006). Over-expression of *ACSL1* was previously associated with increased triglyceride level in mouse liver (Parkes, et al., 2006). Insulin receptor (*INSR*), coding for transmembrane receptor stimulating triacylglycerol synthesis in muscle (Dimitriadis, Mitrou, Lambadiari, Maratou & Raptis, 2011) was another down-regulated gene in LRFI pigs. Repression of these two genes, *ACSL1* and *INSR*, is in agreement with reduced IMF content in meat from LRFI pigs.

Previous studies reported reduced mitochondrial energy metabolism in the *Longissimus* muscle of low vs high RFI pigs (Fu, et al., 2017; Jing, et al., 2015; Le Naou, et al., 2012). In this study, functional annotation revealed ‘oxidation of fatty acids’ significantly over-represented amongst DE genes with the direction towards inhibition in LRFI pigs (z-score = -1.12), which suggests that the LRFI pigs might exhibit reduced mitochondrial energy metabolism. Similarly, enrichment of DE genes in lipid catabolic processes was previously reported in RFI divergent pigs (Jing, et al., 2015). Moreover, succinate dehydrogenase complex iron sulphur subunit B (*SDHB*), involved in complex II of the mitochondrial electron transport chain (Liu, et al., 2015), and transcription factor A mitochondrial (*TFAM*), a key modulator of mitochondrial DNA replication and transcription (Zou, et al., 2016), were down-regulated in low RFI pigs.

5. Conclusions

To conclude, improvement in feed efficiency revealed meat quality characteristics generally within the normal range for the production of acceptable quality pork. However significant differences in traits such as sensory profile, texture, and technological aspects such as cook loss suggest there is a minor impairment of meat quality in more feed efficient pigs, at least in the *M. longissimus*. High FE pigs were also associated with leaner carcasses, greater muscle content and enhanced nutritional value in terms of fatty acid composition. Gene expression profiling of muscle from RFI-divergent pigs provided mechanistic insights on the biological events governing differences in RFI that have consequences for eating quality. A number of differentially expressed genes were significantly over-represented with functions in muscle growth & development, lipid metabolism and connective tissue metabolism. Up-regulation of genes involved in the synthesis and degradation of protein suggest a greater muscle protein turnover in low RFI pigs, while the divergence in adhesion of connective tissue may contribute to differences in tenderness. Moreover, a tendency towards suppression of fatty acid oxidation and down-regulation of *SDHB* and *TFAM* could possibly contribute to reduced mitochondrial activity in low RFI muscle.

Acknowledgements

The authors wish to thank Carol Griffin for training sensory panellists and conducting sensory assessment of the samples, as well as staff and students at Teagasc in Ashtown who assisted in animal sampling. The authors also thank Hannelore Tychsen for her excellent technical help. The ECO-FCE project was funded by the European Union Seventh Framework Programme (FP7 2007/2013) under grant agreement No. 311794.

Supplementary data

Table S1 Growth performance parameters of the pigs (10 low and 10 high RFI) selected for meat quality and transcriptomic analysis.

Table S2 Forward and reverse primers for microarray validation through qPCR.

Table S3 Differentially expressed probe sets (n=1,035) between LRFI and HRFI groups.

Table S4 Molecular and cellular functions significantly over-represented among differentially expressed genes including list of molecules contained within each function.

Table S5 Differentially expressed miRNAs in relation to RFI and the number of targeted differentially expressed transcripts.

Figure S1 Network #1(miRNA target genes): cellular development, growth and proliferation, respiratory system development and function.

References

- Adams, C., Cazzanelli, G., Rasul, S., Hitchinson, B., Hu, Y., Coombes, R. C., . . . Yague, E. (2015). Apoptosis inhibitor TRIAP1 is a novel effector of drug resistance. *Oncol Rep*, 34(1), 415-422.
- Alzhanov, D. T., McInerney, S. F. & Rotwein, P. (2010). Long Range Interactions Regulate Igf2 Gene Transcription during Skeletal Muscle Differentiation. *Journal of Biological Chemistry*, 285(50), 38969-38977.
- Aslan, O., Hamill, R. M., Davey, G., McBryan, J., Mullen, A. M., Gispert, M. & Sweeney, T. (2012). Variation in the IGF2 gene promoter region is associated with intramuscular fat content in porcine skeletal muscle. *Molecular Biology Reports*, 39(4), 4101-4110.
- Biasiotta, A., D'Arcangelo, D., Passarelli, F., Nicodemi, E. M. & Facchiano, A. (2016). Ion channels expression and function are strongly modified in solid tumors and vascular malformations. *J Transl Med*, 14(1), 285.
- Birch, G. G. & Kemp, S. E. (1989). Apparent specific volumes and tastes of amino-acids. *Chemical Senses*, 14(2), 249-258.
- Bryhni, E. A., Byrne, D. V., Rodbotten, M., Moller, S., Claudi-Magnussen, C., Karlsson, A., . . . Martens, M. (2003). Consumer and sensory investigations in relation to physical/chemical aspects of cooked pork in Scandinavia. *Meat Science*, 65(2), 737-748.
- Bumgarner, R. (2013). DNA microarrays: Types, Applications and their future. *Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.]*, 0 22, Unit-22.21.
- Cánovas, A., Quintanilla, R., Amills, M. & Pena, R. N. (2010). Muscle transcriptomic profiles in pigs with divergent phenotypes for fatness traits. *BMC Genomics*, 11(1), 372.
- Choudhuri, S. P., Delay, R. J. & Delay, E. R. (2015). L-Amino Acids Elicit Diverse Response Patterns in Taste Sensory Cells: A Role for Multiple Receptors. *Plos One*, 10(6).
- Cruzen, S. M., Harris, A. J., Hollinger, K., Punt, R. M., Grubbs, J. K., Selsby, J. T., . . . Huff-Lonergan, E. (2013). Evidence of decreased muscle protein turnover in gilts selected for low residual feed intake. *J Anim Sci*, 91(8), 4007-4016.
- Dimitriadis, G., Mitrou, P., Lambadiari, V., Maratou, E. & Raptis, S. A. (2011). Insulin effects in muscle and adipose tissue. *Diabetes Res Clin Pract*, 93 Suppl 1, S52-59.
- Dominguez, R. & Lorenzo, J. M. (2014). Effect of genotype on fatty acid composition of intramuscular and subcutaneous fat of Celta pig breed. *Grasas Y Aceites*, 65(3).
- Dugan, M. E. R., Vahmani, P., Turner, T. D., Mapiye, C., Juárez, M., Prieto, N., . . . Aalhus, J. L. (2015). Pork as a Source of Omega-3 (n-3) Fatty Acids. *Journal of Clinical Medicine*, 4(12), 1999-2011.
- Fan, B., Lkhagvadorj, S., Cai, W., Young, J., Smith, R. M., Dekkers, J. C. M., . . . Rothschild, M. F. (2010). Identification of genetic markers associated with residual feed intake and meat quality traits in the pig. *Meat Science*, 84(4), 645-650.
- Faure, J., Lefaucheur, L., Bonhomme, N., Ecolan, P., Météau, K., Coustard, S. M., . . . Lebret, B. (2013). Consequences of divergent selection for residual feed intake in pigs on muscle energy metabolism and meat quality. *Meat Science*, 93(1), 37-45.
- Freeman, T. C., Ivens, A., Baillie, J. K., Beraldi, D., Barnett, M. W., Dorward, D., . . . Hume, D. A. (2012). A gene expression atlas of the domestic pig. *BMC Biol*, 10, 90.
- Fu, L. L., Xu, Y. Y., Hou, Y., Qi, X. L., Zhou, L., Liu, H. Y., . . . Li, X. Y. (2017). Proteomic analysis indicates that mitochondrial energy metabolism in skeletal muscle tissue is negatively correlated with feed efficiency in pigs. *Scientific Reports*, 7.

- Geesink, G. H., Taylor, R. G. & Koohmaraie, M. (2005). Calpain 3/p94 is not involved in postmortem proteolysis. *J Anim Sci*, 83(7), 1646-1652.
- Gilbert, H., Bidanel, J. P., Gruand, J., Caritez, J. C., Billon, Y., Guillouet, P., . . . Sellier, P. (2007). Genetic parameters for residual feed intake in growing pigs, with emphasis on genetic relationships with carcass and meat quality traits. *J Anim Sci*, 85(12), 3182-3188.
- Git, A., Dvinge, H., Salmon-Divon, M., Osborne, M., Kutter, C., Hadfield, J., . . . Caldas, C. (2010). Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. *Rna*, 16(5), 991-1006.
- Gondret, F., Vincent, A., Houee-Bigot, M., Siegel, A., Lagarrigue, S., Causeur, D., . . . Louveau, I. (2017). A transcriptome multi-tissue analysis identifies biological pathways and genes associated with variations in feed efficiency of growing pigs. *BMC Genomics*, 18(1), 244.
- Honikel, K. O. (1998). Reference methods for the assessment of physical characteristics of meat. *Meat Science*, 49(4), 447-457.
- Hoque, M. A. & Suzuki, K. (2009). Genetics of Residual Feed Intake in Cattle and Pigs: A Review. *Asian-Australasian Journal of Animal Sciences*, 22(5), 747-755.
- Jing, L., Hou, Y., Wu, H., Miao, Y. X., Li, X. Y., Cao, J. H., . . . Zhao, S. H. (2015). Transcriptome analysis of mRNA and miRNA in skeletal muscle indicates an important network for differential Residual Feed Intake in pigs. *Scientific Reports*, 5.
- Kasprzyk, A., Tyra, M. & Babicz, M. (2015). Fatty acid profile of pork from a local and a commercial breed. *Arch. Anim. Breed.*, 58(2), 379-385.
- Kawai, M., Sekine-Hayakawa, Y., Okiyama, A. & Ninomiya, Y. (2012). Gustatory sensation of (L)- and (D)-amino acids in humans. *Amino Acids*, 43(6), 2349-2358.
- Kurisaki, T., Masuda, A., Sudo, K., Sakagami, J., Higashiyama, S., Matsuda, Y., . . . Sehara-Fujisawa, A. (2003). Phenotypic analysis of Meltrin alpha (ADAM12)-deficient mice: Involvement of Meltrin alpha in adipogenesis and myogenesis. *Mol Cell Biol*, 23(1), 55-61.
- Le Naou, T., Le Floc'h, N., Louveau, I., Gilbert, H. & Gondret, F. (2012). Metabolic changes and tissue responses to selection on residual feed intake in growing pigs. *J Anim Sci*, 90(13), 4771-4780.
- Lefaucheur, L., Lebret, B., Ecolan, P., Louveau, I., Damon, M., Prunier, A., . . . Gilbert, H. (2011). Muscle characteristics and meat quality traits are affected by divergent selection on residual feed intake in pigs. *Journal of Animal Science*, 89(4), 996-1010.
- Littler, D. R., Harrop, S. J., Goodchild, S. C., Phang, J. M., Mynott, A. V., Jiang, L., . . . Curmi, P. M. (2010). The enigma of the CLIC proteins: Ion channels, redox proteins, enzymes, scaffolding proteins? *FEBS Lett*, 584(10), 2093-2101.
- Liu, X., Du, Y., Trakooljul, N., Brand, B., Muryni, E., Krschek, C., . . . Ponsuksili, S. (2015). Muscle Transcriptional Profile Based on Muscle Fiber, Mitochondrial Respiratory Activity, and Metabolic Enzymes. *International Journal of Biological Sciences*, 11(12), 1348-1362.
- Lobley, G. E. (2003). Protein turnover—what does it mean for animal production? *Canadian Journal of Animal Science*, 83(3), 327-340.
- Lui, H. M., Chen, J., Wang, L. L. & Naumovski, L. (2003). ARMER, apoptotic regulator in the membrane of the endoplasmic reticulum, a novel inhibitor of apoptosis. *Molecular Cancer Research*, 1(7), 508-518.
- McBryan, J., Hamill, R. M., Davey, G., Lawlor, P. & Mullen, A. M. (2010). Identification of suitable reference genes for gene expression analysis of pork meat quality and

- analysis of candidate genes associated with the trait drip loss. *Meat Science*, 86(2), 436-439.
- McCormick, R. J. (1994). The flexibility of the collagen compartment of muscle. *Meat Sci*, 36(1-2), 79-91.
- Metzler-Zebell, B. U., Lawlor, P. G., Magowan, E., McCormack, U. M., Curiao, T., Hollmann, M., . . . Zebell, Q. (2017). Finishing pigs that are divergent in feed efficiency show small differences in intestinal functionality and structure. *Plos One*, 12(4).
- Moghadaszadeh, B., Albrechtsen, R., Guo, L. T., Zaik, M., Kawaguchi, N., Borup, R. H., . . . Wewer, U. M. (2003). Compensation for dystrophin-deficiency: ADAM12 overexpression in skeletal muscle results in increased $\alpha 7$ integrin, utrophin and associated glycoproteins. *Human Molecular Genetics*, 12(19), 2467-2479.
- Nazarov, P. V., Muller, A., Kaoma, T., Nicot, N., Maximo, C., Birembaut, P., . . . Vallar, L. (2017). RNA sequencing and transcriptome arrays analyses show opposing results for alternative splicing in patient derived samples. *BMC Genomics*, 18(1), 443.
- 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. *Lipid Peroxidation*, 239-258.
- Norris, A. W., Chen, L. H., Fisher, S. J., Szanto, I., Ristow, M., Jozsi, A. C., . . . Kahn, C. R. (2003). Muscle-specific PPAR gamma-deficient mice develop increased adiposity and insulin resistance but respond to thiazolidinediones. *Journal of Clinical Investigation*, 112(4), 608-618.
- Olsson, V. & Pickova, J. (2005). The influence of production systems on meat quality, with emphasis on pork. *Ambio*, 34(4-5), 338-343.
- Orang, A. V., Safaralizadeh, R. & Kazemzadeh-Bavili, M. (2014). Mechanisms of miRNA-Mediated Gene Regulation from Common Downregulation to mRNA-Specific Upregulation. *International Journal of Genomics*.
- Ouali, A., Herrera-Mendez, C. H., Coulis, G., Becila, S., Boudjellal, A., Aubry, L. & Sentandreu, M. A. (2006). Revisiting the conversion of muscle into meat and the underlying mechanisms. *Meat Science*, 74(1), 44-58.
- Parkes, H. A., Preston, E., Wilks, D., Ballesteros, M., Carpenter, L., Wood, L., . . . Cooney, G. J. (2006). Overexpression of acyl-CoA synthetase-1 increases lipid deposition in hepatic (HepG2) cells and rodent liver in vivo. *American Journal of Physiology-Endocrinology and Metabolism*, 291(4), E737-E744.
- Ponsuksili, S., Jonas, E., Murani, E., Phatsara, C., Srikanthai, T., Walz, C., . . . Wimmers, K. (2008). Trait correlated expression combined with expression QTL analysis reveals biological pathways and candidate genes affecting water holding capacity of muscle. *BMC Genomics*, 9.
- Porrello, A., Cerone, M. A., Coen, S., Gurtner, A., Fontemaggi, G., Cimino, L., . . . Soddu, S. (2000). p53 regulates myogenesis by triggering the differentiation activity of pRb. *J Cell Biol*, 151(6), 1295-1304.
- Przewozniak, M., Czaplicka, I., Czerwinska, A. M., Markowska-Zagrajek, A., Moraczewski, J., Streminska, W., . . . Brzoska, E. (2013). Adhesion Proteins - An Impact on Skeletal Myoblast Differentiation. *Plos One*, 8(5).
- Purslow, P. P. (2014). New developments on the role of intramuscular connective tissue in meat toughness. *Annu Rev Food Sci Technol*, 5, 133-153.
- Rajewsky, N. (2006). microRNA target predictions in animals. *Nature Genetics*, 38, S8-S13.
- Rodier, M. H., El Moudni, B., Kauffmann-Lacroix, C., Daniault, G. & Jacquemin, J. L. (1999). A *Candida albicans* metallopeptidase degrades constitutive proteins of extracellular matrix. *Fems Microbiology Letters*, 177(2), 205-210.

- Saintilan, R., Merour, I., Brossard, L., Tribout, T., Dourmad, J. Y., Sellier, P., . . . Gilbert, H. (2013). Genetics of residual feed intake in growing pigs: Relationships with production traits, and nitrogen and phosphorus excretion traits. *Journal of Animal Science*, *91*(6), 2542-2554.
- Schwab, C. R., Baas, T. J., Stalder, K. J. & Mabry, J. W. (2006). Effect of long-term selection for increased leanness on meat and eating quality traits in Duroc swine. *J Anim Sci*, *84*(6), 1577-1583.
- Smith, R. M., Gabler, N. K., Young, J. M., Cai, W., Boddicker, N. J., Anderson, M. J., . . . Lonergan, S. M. (2011). Effects of selection for decreased residual feed intake on composition and quality of fresh pork. *Journal of Animal Science*, *89*(1), 192-200.
- Storey, J. D. & Tibshirani, R. (2003). Statistical significance for genomewide studies. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(16), 9440-9445.
- Tamir, Y. & Bengal, E. (1998). p53 Protein is activated during muscle differentiation and participates with MyoD in the transcription of muscle creatine kinase gene. *Oncogene*, *17*(3), 347-356.
- Tang, Z., Yang, Y., Wang, Z., Zhao, S., Mu, Y. & Li, K. (2015). Integrated analysis of miRNA and mRNA paired expression profiling of prenatal skeletal muscle development in three genotype pigs. *Scientific Reports*, *5*, 15544.
- Tian, Y., Chang, J. C., Fan, E. Y., Flajolet, M. & Greengard, P. (2013). Adaptor complex AP2/PICALM, through interaction with LC3, targets Alzheimer's APP-CTF for terminal degradation via autophagy. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(42), 17071-17076.
- Van Laere, A.-S., Nguyen, M., Braunschweig, M., Nezer, C., Collette, C., Moreau, L., . . . Andersson, L. (2003). A regulatory mutation in IGF2 causes a major QTL effect on muscle growth in the pig. *Nature*, *425*(6960), 832-836.
- Vincent, A., Louveau, I., Gondret, F., Trefeu, C., Gilbert, H. & Lefaucheur, L. (2015). Divergent selection for residual feed intake affects the transcriptomic and proteomic profiles of pig skeletal muscle. *J Anim Sci*, *93*(6), 2745-2758.
- Wang, X., Chen, J., Liu, H., Xu, Y., Wang, X., Xue, C., . . . Jiang, Z. (2008). The pig p160 co-activator family: Full length cDNA cloning, expression and effects on intramuscular fat content in Longissimus Dorsi muscle. *Domestic Animal Endocrinology*, *35*(2), 208-216.
- Wanitchakool, P., Ousingsawat, J., Sirianant, L., MacAulay, N., Schreiber, R. & Kunzelmann, K. (2016). Cl- channels in apoptosis. *Eur Biophys J*, *45*(7), 599-610.
- Weston, A. R., Rogers, R. W. & Althen, T. G. (2002). Review: The Role of Collagen in Meat Tenderness. *The Professional Animal Scientist*, *18*(2), 107-111.
- Wilkinson, J. M. (2011). Re-defining efficiency of feed use by livestock. *Animal*, *5*(7), 1014-1022.
- Woessner, J. F. (1991). Matrix metalloproteinases and their inhibitors in connective-tissue remodeling. *Faseb Journal*, *5*(8), 2145-2154.
- Won, S. G. L. (2012). Acute and chronic heat stress alters the metabolic profile of skeletal muscle in growing swine. *Master's thesis*.
- Wood, J. D., Enser, M., Fisher, A. V., Nute, G. R., Sheard, P. R., Richardson, R. I., . . . Whittington, F. M. (2008). Fat deposition, fatty acid composition and meat quality: A review. *Meat Science*, *78*(4), 343-358.
- Zou, T. D., Yu, B., Yu, J., Mao, X. B., Zheng, P., He, J., . . . Chen, D. W. (2016). Moderately decreased maternal dietary energy intake during pregnancy reduces fetal skeletal muscle mitochondrial biogenesis in the pigs. *Genes and Nutrition*, *11*.

Table 1 Carcass and technological meat quality traits of LTL muscle from pigs divergent in RFI.

Trait	Low RFI ¹	High RFI ¹	SE	P-value
Fat depth (mm)	11.9	13.3	0.71	0.086
Muscle depth (mm)	53.4	50.9	1.10	0.045
Lean (%)	58.0	56.5	0.64	0.042
pH 45m	6.72	6.58	0.06	0.055
pH 3h	6.71	6.60	0.11	0.296
pH 24h	5.55	5.62	0.09	0.452
L*	53.0	54.2	1.44	0.423
a*	5.43	5.24	0.52	0.725
b*	12.9	13.9	0.34	0.013
1h L*	52.7	54.3	1.57	0.338
1h a*	6.63	5.99	0.56	0.279
1h b*	13.9	14.5	0.38	0.137
DL (%)	2.36	2.31	0.41	0.905
Protein (%)	23.8	23.7	0.30	0.615
Ash (%)	1.31	1.34	0.07	0.704
Moisture (%)	74.9	74.9	0.17	0.868
IMF (%)	1.05	1.41	0.15	0.042
WBSF day 1 (N)	59.1	39.7	4.14	0.001
WBSF day 7 (N)	48.8	38.8	4.72	0.057
CL day 1 (%)	32.9	31.2	0.97	0.053
CL day 7 (%)	31.9	31.0	0.98	0.399

¹Least square means for each trait.

Table 2 Fatty acid composition in LTL muscle and percentage of fatty acid in intramuscular fat (IMF) of pigs divergent in RFI.

	Fatty Acid	mg fatty acid / 100g meat				% fatty acid in IMF			
		Low RFI ¹	High RFI ¹	SE	P-value	Low RFI ¹	High RFI ¹	SE	P-value
SFA	Myristic C14:0	17.4	25.3	0.003	0.041	1.17	1.23	0.001	0.486
	Palmitic C16:0	314	475	0.057	0.016	22.9	24.1	0.003	0.002
	Stearic C18:0	157	247	0.032	0.017	11.9	12.7	0.005	0.144
	Total SFA	491	748	0.092	0.017	35.9	38.0	0.008	0.020
MUFA	Palmitoleic C16:1	40.5	59.0	0.007	0.031	3.06	2.99	0.002	0.740
	Eicosenoic C20:1	9.89	13.9	0.002	0.056	0.79	0.71	0.001	0.380
	Oleic C18:1 n9	555	799	0.094	0.025	40.7	40.9	0.006	0.721
	Total MUFA	605	872	0.102	0.024	44.7	44.7	0.007	0.992
PUFA	Linoleic C18:2 n6	111	127	0.014	0.258	7.59	6.36	0.003	<0.001
	Alpha-linolenic C18:3 n3	10.3	11.1	0.001	0.539	0.79	0.57	0.001	0.023
	Total PUFA	121	139	0.015	0.265	8.36	6.94	0.003	0.001

¹Least square means for each fatty acid; SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

Table 3 Canonical signalling pathways significantly differentially expressed in relation to RFI.

Ingenuity Canonical Pathways	-log (B-H p-value)	Genes
TR/RXR Activation	4.90	PIK3C2B , UCP2 , <i>PIK3C2A</i> , <i>MDM2</i> , <i>BCL3</i> , <i>NCOA3</i> , <i>EP300</i> , <i>PIK3R3</i> , RXRG , HP , <i>NCOA2</i> , PIK3CG , <i>NCOA1</i> , <i>ACACA</i> , <i>TBLIXR1</i> , <i>THRB</i>
PEDF Signalling	2.35	PPARG , <i>PIK3R3</i> , TP53 , PIK3C2B , <i>GDNF</i> , <i>PIK3C2A</i> , PIK3CG , <i>RHOA</i> , <i>SRF</i> , <i>FAS</i> , <i>BCL2</i>
HIF1 α Signalling	1.54	<i>SLC2A5</i> , <i>PIK3R3</i> , TP53 , PIK3C2B , <i>EGLN1</i> , <i>PIK3C2A</i> , PIK3CG , <i>NCOA1</i> , <i>MDM2</i> , <i>MMP2</i> , <i>EP300</i>
Myc Mediated Apoptosis Signalling	1.54	<i>PIK3R3</i> , TP53 , PIK3C2B , <i>CASP9</i> , <i>PIK3C2A</i> , PIK3CG , <i>FAS</i> , <i>BCL2</i>
Aryl Hydrocarbon Receptor Signalling	1.50	TP53 , TRIP11 , <i>TFF1</i> , <i>MDM2</i> , <i>NCOA3</i> , <i>FAS</i> , <i>EP300</i> , RXRG , <i>HSP90B1</i> , <i>NCOA2</i> , <i>TGFB1</i> , <i>GSTA1</i> , <i>ALDH6A1</i>
Adipogenesis pathway	1.50	PPARG , TP53 , <i>HDAC9</i> , <i>CCNH</i> , <i>SMAD9</i> , <i>TGFB1</i> , <i>SAP30L</i> , <i>CLOCK</i> , <i>SMO</i> , <i>TBLIXR1</i> , <i>MNAT1</i> , <i>FZD7</i>
Integrin Signalling	1.42	<i>ITGAM</i> , <i>PIK3R3</i> , <i>PIK3C2A</i> , PIK3C2B , <i>PARVA</i> , <i>WIPF1</i> , <i>TLN2</i> , <i>CAPN10</i> , <i>WASL</i> , PIK3CG , <i>RHOA</i> , <i>RHOG</i> , <i>FYN</i> , <i>CAPN6</i> , <i>CRK</i> , <i>ITGA8</i>
Docosahexaenoic Acid (DHA) Signalling	1.40	<i>PIK3R3</i> , <i>PIK3C2A</i> , <i>BCL2</i> , CASP9 , PIK3C2B , PIK3CG
Lymphotoxin β Receptor Signalling	1.34	<i>PIK3R3</i> , <i>PIK3C2A</i> , <i>LTA</i> , <i>EP300</i> , CASP9 , PIK3C2B , PIK3CG
IL-12 Signalling and Production in Macrophages	1.34	APOA4 , <i>PIK3R3</i> , <i>NCOA1</i> , MYD88 , <i>TGFB1</i> , <i>PIK3C2A</i> , <i>APOD</i> , <i>STAT6</i> , <i>EP300</i> , PIK3C2B , PPARG , PIK3CG

Up-regulated genes in low RFI pigs are highlighted in bold and down-regulated genes in normal typeface.

Table 4 Comparison of the microarray and qPCR data of the differentially expressed genes selected for downstream validation.

Gene	Microarray fold change	qPCR fold change	Spearman's rho
<i>ACACA</i>	1.80*	1.40	0.80***
<i>ACSL1</i>	1.24*	1.25*	0.56*
<i>BCL2</i>	1.98*	1.40	0.84***
<i>MMP2</i>	1.16*	1.10	0.74***
<i>SDHB</i>	1.29**	1.23*	0.66**
<i>TFAM</i>	1.43***	1.56*	0.75***

*P < 0.05, **P < 0.01, ***P < 0.001; up-regulated genes in low RFI pigs are highlighted in bold and down-regulated genes in normal typeface. *ACACA* - acetyl-CoA carboxylase alpha; *ACSL1* - acyl-CoA synthetase long-chain family member 1; *BCL2* BCL2 apoptosis regulator; *MMP2* - matrix metalloproteinase 2; *SDHB* - succinate dehydrogenase complex iron sulfur subunit B; *TFAM* - transcription factor A, mitochondrial.

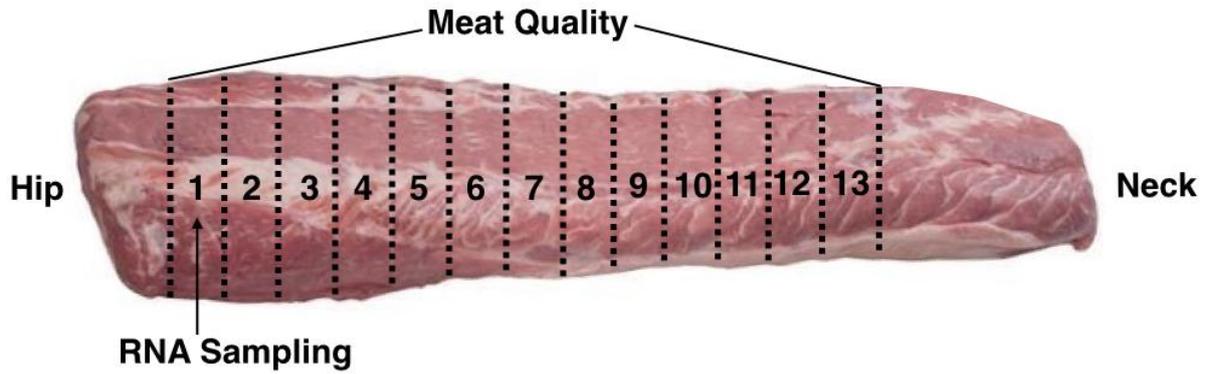


Figure 1 Assignment of *Longissimus thoracis et lumborum* muscle for meat quality measurements (1-13) and transcriptome analysis. 1. Colour, 1-3. Warner Bratzler shear force day 1, 4-6. Warner Bratzler shear force day 7, 7-10. Sensory analysis, 11. Drip loss, 12. Protein, moisture and intramuscular fat content, 13. Fatty acid profile.

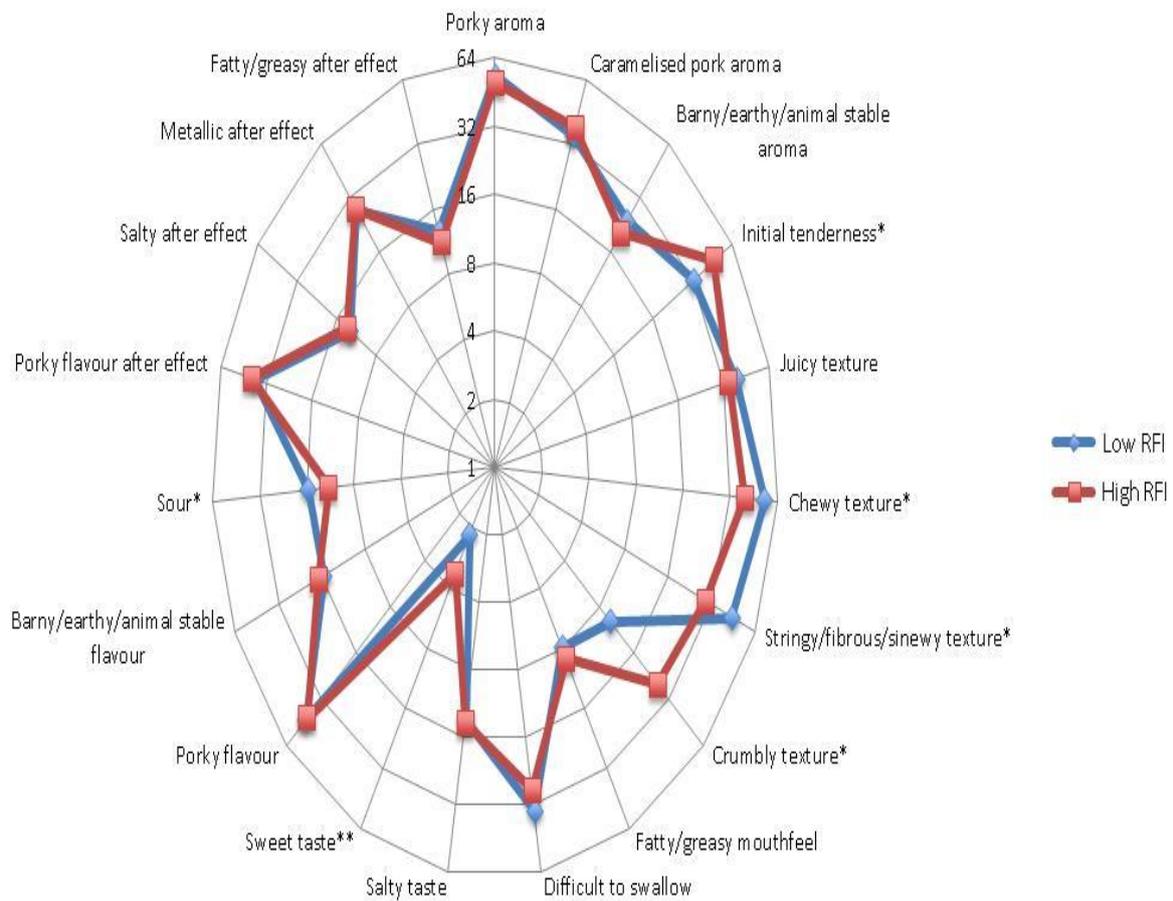


Figure 2 Meat sensory attributes of LTL muscle from low and high RFI pigs (least square means) scored from 0 (not detectable) to 100 (extremely detectable); ** $P < 0.01$, * $P < 0.05$.

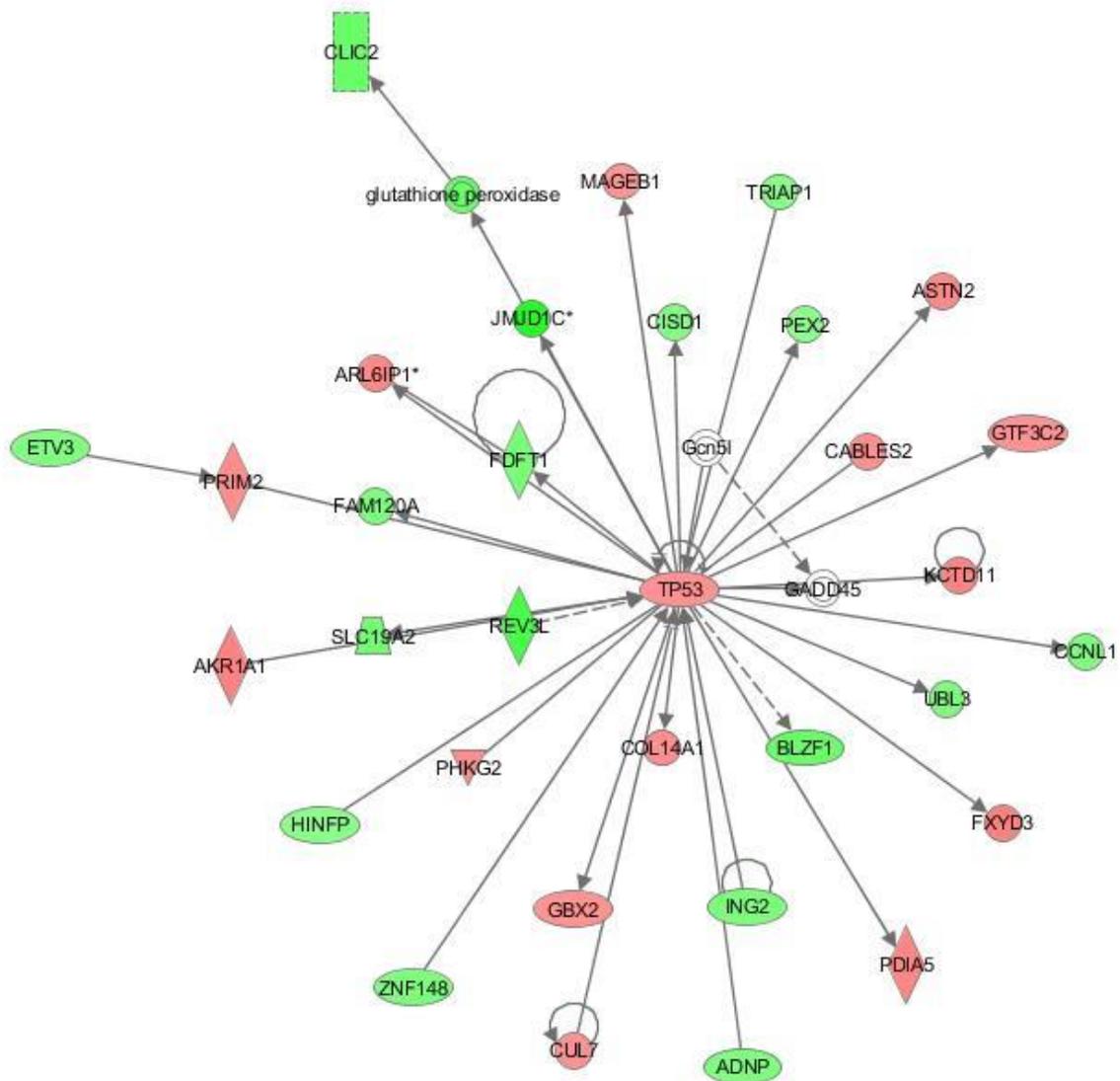


Figure 3 Network #1 (mRNA genes): molecular transport, nucleic acid metabolism and small molecule biochemistry. Genes are denoted as nodes and the biological relationship between two nodes is denoted as an edge/line. Node colour represents up- (red) and down- (green) regulated genes in low RFI pigs.

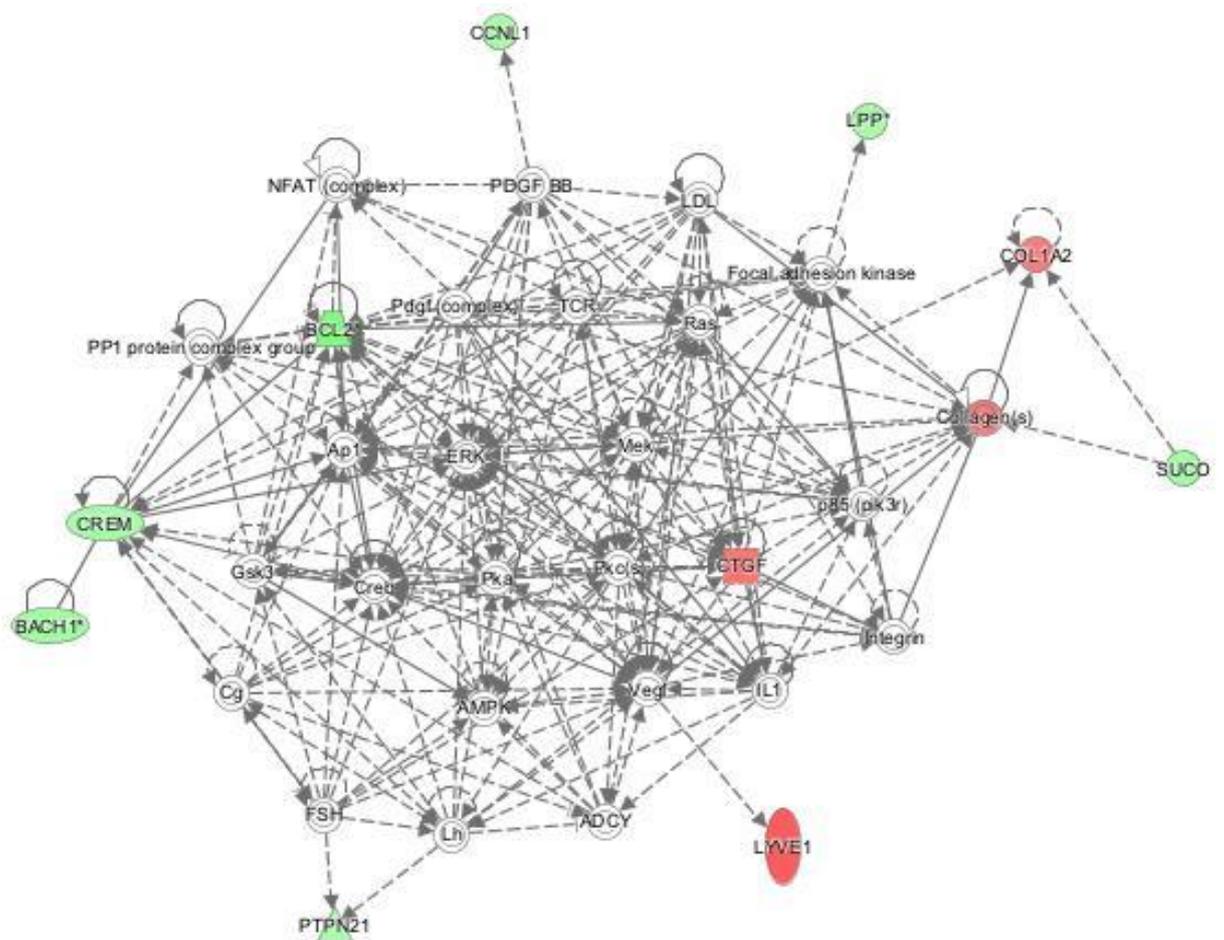


Figure 4 Network #9 (target genes of the miRNAs involved in the network): Embryonic development, connective tissue development and function, organ morphology. Genes are denoted as nodes and the biological relationship between two nodes is denoted as an edge/line. Node colour represents up- (red) and down- (green) regulated genes in low RFI pigs.

Highlights

- Impaired tenderness but improved fatty acid profile in low RFI carcasses.
- Over-expression of growth associated genes in low RFI pigs.
- Differential expression of genes related to connective tissue.

ACCEPTED MANUSCRIPT