

Article

Molecular Mechanisms Underlying Divergent Biochemical Compositions in Longissimus Dorsi of Huainan and Yunan Black Pigs: Insights from Fatty Acids, Amino Acids, and Transcriptomic Profiling

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Abstract

This study investigated meat quality, nutritional characteristics, and transcriptomic regulation in Yunan (YN) black pigs and Huainan (HN) black pigs (n = 6 each). Analysis of fatty acid composition revealed that HN black pigs possessed significantly higher levels of most fatty acids compared to YN black pigs. Notably, the contents of monounsaturated fatty acid C18:1n7c and polyunsaturated fatty acid C18:2n6c in HN black pigs were 1.94-fold and 2.65-fold higher, respectively, than those in YN black pigs. The α -linolenic acid content was also significantly elevated in HN black pigs, indicating an overall higher fatty acid content. Regarding amino acid differences, HN black pigs exhibited significantly higher levels of aspartic acid, glutamic acid, histidine, as well as superior composition of total amino acids, total umami amino acids, and essential amino acids, which contribute to enhanced flavor characteristics and nutritional balance. Transcriptome analysis identified 526 differentially expressed genes in HN vs. YN. KEGG enrichment analysis showed that these genes were involved in many adipogenesis and lipid metabolism signaling pathways, such as biosynthesis of unsaturated fatty acids, fatty acid elongation, apelin signaling pathway and lysine degradation. By integrating transcriptome and protein–protein interaction (PPI) network analyses, we identified key meat quality-related genes: *ELOVL6*, *PRKAG3*, *ROCK2*, and *MYH11*. miRNA profiling identified ssc-miR-133b, ssc-miR-206, and miR-205 as key regulators of meat quality. This study provides a valuable theoretical foundation for understanding the molecular mechanisms underlying pork quality and offers insights for its future improvement.

Keywords: meat quality; amino acid; fatty acid; pigs; mRNA/miRNA



Academic Editor: Javier

Álvarez-Rodríguez

Received: 19 September 2025

Revised: 1 December 2025

Accepted: 4 December 2025

Published: 6 December 2025

Citation: Wen, Y.; Liao, C.; Wang, R.; Wen, B.; Luo, W.; Zhang, W.; Zhu, C.; Sun, H.; Zhang, L.; Liu, X.; et al.

Molecular Mechanisms Underlying Divergent Biochemical Compositions in Longissimus Dorsi of Huainan and Yunan Black Pigs: Insights from Fatty Acids, Amino Acids, and Transcriptomic Profiling. *Agriculture* **2025**, *15*, 2532. <https://doi.org/10.3390/agriculture15242532>

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1. Introduction

In the livestock industry, pork serves as a critical source of high-quality animal protein for human diets, and its quality directly impacting consumer health and satisfaction. The Yunan (YN) black pig and Huainan (HN) black pig, as two excellent indigenous Chinese breeds, have taken their place in the market with their unique meat quality and flavor [1,2]. The HN pig, primarily raised in the Xinyang region of Henan Province, China, is known for its fine muscle fibers, bright red meat color, and high intramuscular fat content, though it has a carcass lean meat percentage of only approximately 44.3% [1,3]. The YN black pig, developed by crossbreeding HN pigs with Duroc pigs, exhibits desirable traits such as rapid growth, high lean meat yield, strong adaptability to roughage, disease resistance, and favorable meat flavor [4]. However, as consumer demand for high-quality pork continues to rise, improving the meat quality of these breeds through scientific approaches has become a key focus in animal science research.

Meat quality is a complex trait that is jointly influenced by multiple genes, the environment and their interactions. Among the key regulators, microRNAs (miRNAs) post-transcriptionally modulate the expression of meat quality-related genes by promoting target mRNA degradation or translational repression [5,6]. As the direct products of gene expression, mRNA levels reflect transcriptional activity under different conditions and are closely associated with meat trait formation. Long non-coding RNAs (lncRNAs), which typically exceed 200 nucleotides in length, regulate gene expression through diverse mechanisms such as chromatin remodeling, transcriptional interference, and miRNA sponging, also playing important roles in meat quality-related regulatory networks [7,8]. For example, Wang et al. performed a comparative transcriptome analysis of mRNAs, miRNAs and lncRNAs in the longest dorsal muscles of lean and fat pigs, and identified a number of up-regulated DE mRNAs, including *LDHB*, *GALNT15*, and *FKBP5*, as well as their interacting DE miRNAs (Differentially expressed miRNAs) and lncRNAs, associated with intramuscular fat deposition [9]. Ma et al. identified traits and loci associated with intramuscular fat content and feed conversion traits for artificial selection in Duroc pigs [10]. Additionally, several studies have explored selection traits in other indigenous Chinese pig breeds, including Anqing Six-End White, Saba, Baoshan, Tunchang, Ding'an, Meishan, and Lulai pigs [11–13]. However, transcriptomic differences between HN and YN black pigs remain uncharacterized. Therefore, transcriptome analysis to resolve miRNA and mRNA networks associated with meat quality in these breeds is essential for elucidating the molecular basis of their meat quality formation.

Fatty acids are key determinants of meat flavor and nutritional value, and their composition and content are regulated by gene expression [14]. Variation in fatty acid content among pig breeds influences not only pork taste and flavor but also its nutritional quality [15,16]. For instance, Duroc pigs were reported to exhibit higher intramuscular concentrations of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), but lower polyunsaturated fatty acid (PUFA) levels, compared to Landrace pigs [17]. Similarly, Zhang et al. analyzed fatty acid composition in the longissimus dorsi muscle of eight purebred pig breeds and found that Duroc pigs had the highest total SFA content, Poland China pigs had a higher MUFA content than all other breeds except Spotted, and Hampshire, Landrace and Yorkshire pigs had a higher PUFA content than the other breeds [14]. Moreover, amino acid composition critically influences the nutritional value and sensory attributes of pork, such as taste and aroma. Pork is rich in essential amino acids, including lysine, threonine, methionine, and tryptophan, which account for approximately 40% of the total amino acids [18]. The balance of these amino acids affects protein bioavailability. Furthermore, specific free amino acids—such as glutamic acid, aspartic acid, glycine, and alanine—and their derivatives are major contributors to umami and sweet flavors, playing a

decisive role in the overall flavor profile of pork [19]. Therefore, analyzing gene expression networks related to meat quality in YN and HN black pigs, along with assessing differences in fatty acid and amino acid composition, will enable a more comprehensive understanding of the genetic basis of their meat quality traits. By exploring the regulatory roles of miRNAs and mRNAs in meat quality formation and their association with nutrient composition, this study aims to reveal the genetic mechanisms underlying meat quality characteristics in YN and HN black pigs and provide a scientific basis for genetic improvement and industrial development of these breeds.

2. Materials and Methods

2.1. Ethics Approval

All animal studies were conducted in accordance with the experimental practices and standards of the Animal Welfare and Research Ethics Committee of Henan Institute of Science and Technology (No. 2024667) issued on 7 March 2022.

2.2. Animals and Tissue Samples

Six each of HN black pigs and YN black pigs were randomly selected from Henan Sangao Agriculture and Animal Husbandry Co., Ltd. (Xinyang, China) and kept under the same rearing conditions and environment for 190 days. The experimental diet consisted of corn (58%), soybean meal (14%), wheat bran (9%), grass meal (15%), and premix (4%). The diet had a digestible energy content of 11.33 MJ/kg and a crude protein content of 13.13%. Based on the standardized composition of the diet and published nutritional data for the ingredients, the calculated dietary fatty acid profile was predominantly linoleic acid (C18:2n6c), consistent with the high corn and soybean meal content. Pigs were temporarily housed in environmentally controlled facilities maintained at 20 ± 2 °C with natural lighting and relative humidity of 60–70%. The ventilation system provided ≥ 15 air changes per hour, while ammonia concentrations were kept below 15 ppm. We divided these pigs into two groups: HN and YN ($n = 6$). The pigs had free access to food and water and were kept in the same conditions throughout the entire trial period. To ensure an identical metabolic state at slaughter, all pigs were subjected to a standardized overnight fast (12 h) with free access to water prior to the procedure. To minimize animal suffering, pigs were weighed and electroshocked before death. All slaughtering procedures were conducted within a single continuous block on the same day, with individuals from the HN and YN groups processed in a randomized order to eliminate any potential batch effects. Head-only stunning was applied to the temporal region at 200–400 V, 1.25–2.5 A for 1–3 s to ensure immediate unconsciousness. Immediately after slaughter, dorsal longissimus muscle samples from each pig were collected into 2 mL freezing tubes (Corning, New York, NY, USA). LD muscle samples for RNA extraction were immediately frozen in liquid nitrogen and stored at -80 °C. Samples for fatty acid composition determination were stored in -80 °C until analysis.

2.3. Determination of Fatty Acid Content

Fatty acid content was analyzed as their methyl ester derivatives via gas chromatography-mass spectrometry (GC-MS). Briefly, lipids were extracted from the samples with organic solvents, transesterified with methanolic H_2SO_4 , and the derived FAMES were analyzed using an Agilent GC-MS system equipped with a CP-Sil 88 column (Agilent Technologies, Inc., Santa Clara, CA, USA).

2.4. Characterization and Quantification of Fatty Acids

Each peak in the total ion flow chromatogram was searched against mass spectral data for known substances in the NIST, Wiley, and Menalib databases. Each peak in the total ion flow chromatogram was identified based on a match of >800. Further screening and characterization were performed in conjunction with the peak times and contents of the fatty acid standards, and the quantification of the substances was replaced by the peak area and divided by the sample mass.

2.5. *Longissimus Dorsi* Muscle

0.5 g muscle samples were homogenized with 5 mL 0.01 M hydrochloric acid and centrifuged at 5000 rpm for 5 min, then 0.5 mL supernatants were mixed with 8% salicyl sulfonic acid for one night under 4 °C. The mixtures were further centrifuged at 12,000 rpm for 10 min for twice. The final supernatants were used for amino acids analysis using Highspeed Amino Acid Analyzer L8900 (Hitachi, Ltd., Tokyo, Japan).

2.6. Total RNA Isolation and Sequencing

Total RNA of the samples was extracted using standard extraction methods, and strict quality control ($OD_{260/280} = 1.8\text{--}2.2$, $OD_{260/230} \geq 2.0$, $RIN \geq 6.5$, $28S:18S \geq 1.0$) of the RNA samples was performed using agarose gel electrophoresis, NanoPhotometer Spectrophotometer, and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), including detection of the concentration, purity, and integrity of the RNA samples. The cDNAs were synthesized for RT-qPCR using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Beijing, China). Approximately 5 µg of total RNAs per sample was used for sequencing library preparation using a NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA). The ribosomal RNAs were removed using a Ribo-Zero™ Gold-Kits (Epicentre, Madison, WI, USA). The RNAs were fragmented and reverse transcribed using the TruSeq RNA LT/HT Sample Preparation Kit (San Diego, CA, USA). The library preparations were sequenced on an Illumina HiSeq™ 2000 sequencing platform after the quality of the cDNA libraries were qualified by Bioanalyzer 2200 evaluation (Agilent, Santa Clara, CA, USA).

For miRNA sequencing, small RNAs (18–30 nt) were gel-purified from total RNA. The NEBNext Multiplex Small RNA Library Prep Kit (NEB) was used for 3' and 5' adapter ligation, reverse transcription, and PCR amplification. Resulting libraries (140–160 bp) were quality-controlled via Agilent Small RNA Kit and sequenced as 75 bp single-end reads on Illumina NextSeq 550 at 15 million reads per sample.

2.7. Bioinformatic Analysis of mRNA and miRNA

The raw sequencing data of mRNA and miRNA libraries were filtered using SOAP-nuke 1.5.2. In this step, reads containing sequencing adapters, low-quality reads (base quality less than or equal to 5), and unknown bases ('N' bases) were removed. After filtering, clean reads were obtained and stored in FASTQ format. Clean reads were mapped to the porcine reference genome (Sus scrofa 11.1). Mapping clean labels of miRNAs to the reference genome using Bowtie2. FPKM (reads per kilo base of the exon model per million mapped reads) values obtained using Cufflinks 2.1.1 were used as values for normalized gene expression and annotated with the NCBI genome assembly. Statistically significant differentially expressed genes (DEGs) and miRNAs were obtained using DESeq2 software (v1.36) with a q value threshold of <0.05 and $|\log_2(\text{foldchange})| > 1$. Principal component analysis (PCA) was used to evaluate the differences between groups and the duplication of samples within groups. Linear algebra calculation methods were used to reduce the dimension and extract principal components of tens of thousands of genetic

variables, and then a PCA graph was drawn. ClusterProfiler software (version 4.0) was used to perform GO functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on the differential gene sets. The miRNA–mRNA interactions were predicted using an integrated approach to enhance reliability. Use miRDB and TargetScan databases to predict the interactions between miRNAs and mRNAs. Only miRNA–mRNA pairs consistently identified by both databases were retained for subsequent analysis.

2.8. Protein–Protein Interaction Analysis (PPI) of DEGs

The PPI network for innate immunity-related DEGs was constructed using the STRING database (<http://string-db.org>, accessed on 15 September 2025). STRING provides known and predicted PPIs, encompassing both direct and indirect associations derived from genomic context, high-throughput experiments, and curated biological knowledge. After obtaining the PPI relationships (set confidence > 0.400 while retaining default values for all other parameters), the network graph was constructed using Cytoscape (version 3.7.2).

2.9. Quantitative Real-Time PCR (qRT-PCR) Validation of DEGs and miRNAs

To validate the RNA-seq results, six differentially expressed genes and six miRNAs were selected for qRT-PCR analysis. Total RNA was extracted using TRIzol, and cDNA was synthesized using a PrimeScript RT Kit (TaKaRa, Tokyo, Japan) for mRNAs and miScript RT Kit (Qiagen, Hilden, Germany) for miRNAs. Primers for target genes and miRNAs were designed using Primer 5 software (version 5.0). The qRT-PCR reactions were conducted in triplicate using SYBR Green PCR Master Mix, with β -actin, and U6 serving as internal controls for mRNA and miRNA validation, respectively. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

2.10. Statistical Analysis

The fatty acid content was determined using Microsoft Excel, and an unpaired *t*-test (SPSS 22.0) was used for intergroup comparisons. All data are reported as mean $\pm$ standard deviation, with $p < 0.05$ and $p < 0.01$ indicating statistical and high significance, respectively.

3. Results

3.1. Fatty Acid Composition of HN Black Pigs and YN Black Pigs

This study systematically analyzed the contents of 15 fatty acids in the muscles of YN black pigs and HN black pigs. The results showed significant differences between the two breeds (Table 1). HN black pigs had significantly higher contents in most fatty acid types than HN black pigs. Among them, the content differences in key fatty acids such as C15:1, C16:0, C16:1, C18:0, C18:1n9c, and C18:2n6c all reached statistical significance ($p < 0.05$). In particular, for the monounsaturated fatty acid C18:1n9c (oleic acid) and the polyunsaturated fatty acid C18:2n6c (linoleic acid), the contents in HN black pigs were 1.94-fold and 2.65-fold those in YN black pigs, respectively, indicating that HN black pigs had more advantages in lipid nutritional characteristics. In addition, non-significantly different fatty acids such as C14:0 and C17:1 also showed a higher trend in black pigs. It is worth noting that although the absolute content of C18:3n3 (α -linolenic acid) was relatively low, the content in HN black pigs (0.07 ± 0.003) was significantly higher than that in YN black pigs (0.015 ± 0.002 , $p < 0.05$), suggesting that HN black pigs had a better ability to accumulate n-3 series fatty acids. Overall, HN black pigs exhibited richer fatty acid composition characteristics, especially in terms of monounsaturated and polyunsaturated fatty acid contents, which were significantly superior to those of HN black pigs.

Table 1. Fatty acid differences between HN and YN black pigs.

Types of Fatty Acids	HN Black Pigs (ug/mg)	YN Black Pigs (ug/mg)	<i>p</i> -Value
C14:0	0.42 ± 0.08	0.23 ± 0.02	<i>p</i> = 0.34
C15:1	6.47 ± 1.56	3.57 ± 0.43	<i>p</i> = 0.012
C16:0	10.58 ± 1.20	5.83 ± 0.70	<i>p</i> = 0.005
C16:1	1.09 ± 0.35	0.61 ± 0.02	<i>p</i> = 0.021
C17:0	0.04 ± 0.01	0.02 ± 0.002	<i>p</i> = 0.24
C17:1	0.02 ± 0.008	0.01 ± 0.001	<i>p</i> = 0.24
C18:0	4.92 ± 1.24	2.56 ± 0.46	<i>p</i> = 0.023
C18:1n9t	0.07 ± 0.02	0.03 ± 0.004	<i>p</i> = 0.22
C18:1n9c	15.83 ± 5.79	8.17 ± 1.24	<i>p</i> = 0.002
C18:2n6c	2.91 ± 0.25	1.10 ± 0.13	<i>p</i> = 0.019
C20:1	0.16 ± 0.08	0.05 ± 0.01	<i>p</i> = 0.23
C18:3n3	0.07 ± 0.003	0.015 ± 0.002	<i>p</i> = 0.17
C20:2	0.07 ± 0.03	0.02 ± 0.002	<i>p</i> = 0.19
C22:1n9	0.05 ± 0.009	0.04 ± 0.005	<i>p</i> = 0.48
C20:4n6	0.1 ± 0.01	0.05 ± 0.002	<i>p</i> = 0.13

3.2. Amino Acid Differences Between HN and YN Black Pigs

The amino acid composition of HN black pigs exhibited significant superiority over YN Black pigs in multiple critical parameters (Table 2). Notably, HN black pigs demonstrated statistically significant ($p < 0.05$) advantages in aspartic acid, glutamic acid, and histidine, with histidine content reaching 1.74-fold higher than YN black pigs. This genetic advantage was further reflected in total amino acid content and total umami amino acids, particularly through elevated glutamate and aspartate levels. The essential amino acid profile also showed marked improvement, indicating superior protein nutritional value. While non-significant differences were observed for serine and arginine, HN black pigs maintained upward trends in glycine and tyrosine. These findings collectively demonstrate HN black pigs enhanced flavor characteristics and nutritional balance, with histidine and glutamate playing key roles in their distinctive flavor profile.

Table 2. Differences in muscle amino acids between HN and YN black pigs.

Amino Acids	HN Black Pigs (%)	YN Black Pigs (%)	<i>p</i> -Value
Aspartic acid	2.20 ± 0.20	1.96 ± 0.09	<i>p</i> = 0.041
Glutamic acid	3.46 ± 0.29	3.04 ± 0.13	<i>p</i> = 0.049
Serine	0.99 ± 0.08	0.90 ± 0.05	<i>p</i> = 0.54
Arginine	1.50 ± 0.13	1.35 ± 0.06	<i>p</i> = 0.65
Glycine	1.36 ± 0.12	1.19 ± 0.05	<i>p</i> = 0.59
Threonine	1.08 ± 0.10	0.94 ± 0.04	<i>p</i> = 0.72
Proline	0.85 ± 0.08	0.78 ± 0.04	<i>p</i> = 0.69
Alanine	1.26 ± 0.12	1.12 ± 0.05	<i>p</i> = 0.48
Valine	1.22 ± 0.13	1.09 ± 0.06	<i>p</i> = 0.047
Methionine	0.35 ± 0.07	0.32 ± 0.03	<i>p</i> = 0.79
Cystine	0.17 ± 0.02	0.15 ± 0.01	<i>p</i> = 0.81
Isoleucine	1.08 ± 0.09	0.94 ± 0.04	<i>p</i> = 0.65
Leucine	1.80 ± 0.13	1.62 ± 0.09	<i>p</i> = 0.044
Phenylalanine	0.93 ± 0.08	0.83 ± 0.02	<i>p</i> = 0.35
Histidine	1.51 ± 0.09	0.87 ± 0.05	<i>p</i> = 0.021
Lysine	2.47 ± 0.22	2.12 ± 0.08	<i>p</i> = 0.36
Tyrosine	0.76 ± 0.08	0.64 ± 0.02	<i>p</i> = 0.44
Total amino acid	23.00 ± 2.04	19.88 ± 0.89	<i>p</i> = 0.034
Total umami amino acids	18.19 ± 1.89	16.11 ± 1.31	<i>p</i> = 0.029
Total essential amino acids	8.93 ± 1.01	7.86 ± 0.98	<i>p</i> = 0.042

3.3. mRNA-Seq Analysis and DEG Profiling

12 cDNA libraries were analyzed by high-throughput sequencing. Groups HN and YN generated 254,870,368 and 252,910,472 raw reads, respectively. After quality control, 239,107,354 and 237,629,000 clean reads were obtained, with average quality Q20 and Q30 scores of $\geq 96.28\%$ and 90.82% for each library, respectively, and an average GC content of 53.01% . Clean reads were mapped to the pig genome, representing $84.54\text{--}88.63\%$ of the clean reads from the 12 samples (Table 3).

Table 3. Summary of mRNA sequencing data quality and the statistics of the transcriptome assemblies.

Sample	Raw_Reads	Clean_Reads	Q20	Q30	GC (%)	Mapping Rate
HN1	44,556,038	41,516,514	96.32	90.9	51.87	85.41%
HN2	42,522,622	39,570,808	96.52	91.27	51.8	86.46%
HN3	41,716,848	38,210,420	96.77	91.36	53.86	86.25%
HN4	47,065,726	43,197,454	96.28	90.82	51.88	83.85%
HN5	37,247,516	37,021,118	96.36	91.14	54.83	81.47%
HN6	41,761,618	39,591,040	96.92	91.66	52.21	88.08%
YN1	39,774,740	37,323,634	96.47	91.26	53.74	86.12%
YN2	44,155,756	41,376,022	96.92	91.64	52.15	88.63%
YN3	43,360,774	39,728,160	96.36	91.00	53.44	84.54%
YN4	44,279,380	42,238,080	96.79	91.46	53.42	88.27%
YN5	40,122,388	38,217,128	96.55	91.38	53.62	86.96%
YN6	41,217,434	38,745,976	96.32	90.85	53.33	86.57%

Principal component analysis (PCA) of the samples from the HN and YN groups showed that the samples from each group were closely clustered (Figure 1A). The Venn diagram analysis shows the number of DEGs shared by the HN and YN groups as well as the number of DEGs unique to each group (Figure 1B). Using $\text{FDR} < 0.05$ and $|\log_2(\text{FC})| > 1$ as criteria, 526 DEGs were identified in HN vs. YN, of which 261 were up-regulated and 265 were down-regulated (Figure 1C).

3.4. GO Terms and KEGG Pathway Enrichment Analyses

GO and KEGG pathway analysis for studying potential functions of DEGs. GO enrichment revealed that DEGs are mainly annotated in peptide metabolic process, translation, carbohydrate metabolic process, inorganic anion transport, peptide biosynthetic process and amide biosynthetic process in HN vs. YN (Figure 2A). KEGG analysis showed that regulation of actin cytoskeleton, retinol metabolism, apelin signaling pathway were significantly enriched in HN vs. YN (Figure 2B). Crucially, the enrichment of these pathways was driven by key DEGs with established functions in meat quality. For example, the enrichment of the ‘regulation of actin cytoskeleton’ pathway was significantly attributed to *ROCK2* and *MYH11*, genes critical for muscle cell structure and contractility, thereby providing a molecular basis for divergent meat tenderness. Similarly, the significant enrichment of the ‘apelin signaling pathway’, a key regulator of energy metabolism, was underpinned by the differential expression of *PRKAG3*, which aligns perfectly with the observed variations in intramuscular fat deposition between the two breeds.

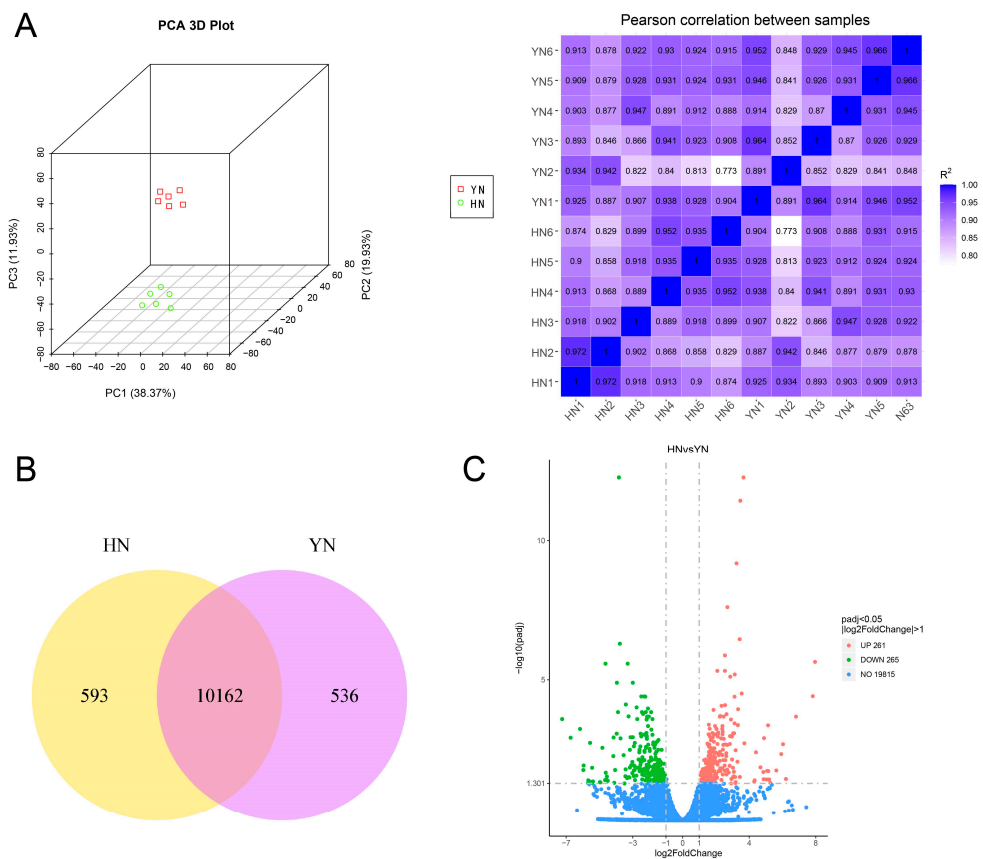


Figure 1. Analysis of sample relationships and DEGs after RNA-seq. (YN) Yunan Black pigs. (HN) Huainan Black pigs. **(A)** Principal Component Analysis (PCA) and correlation analysis of samples. **(B)** Venn diagram showing DEGs overlap for each group. **(C)** Volcano map of DEGs.

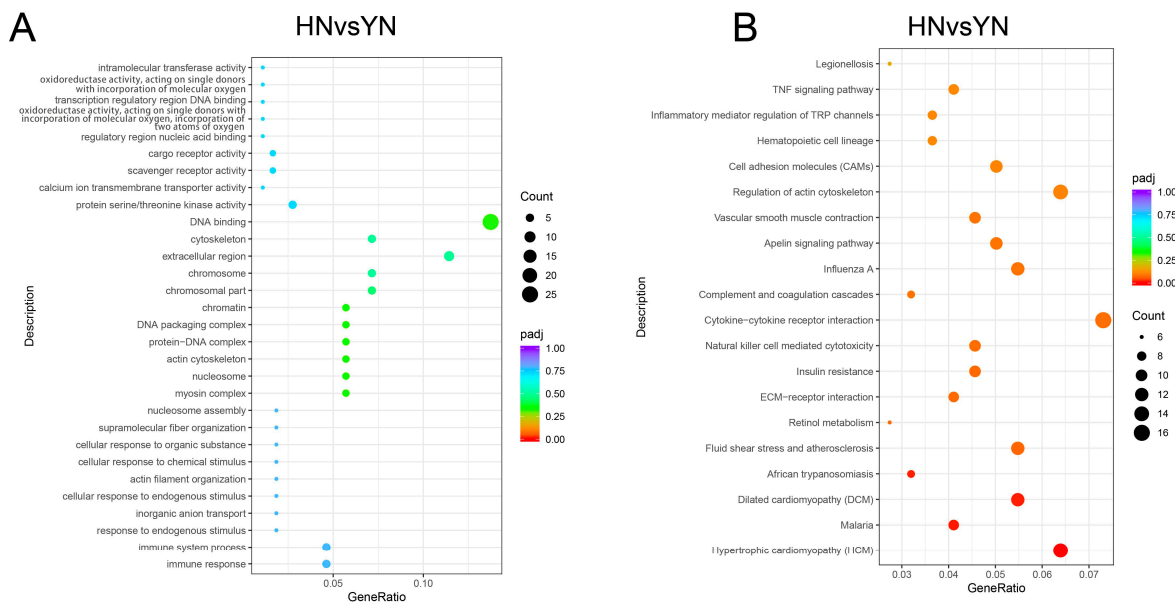


Figure 2. GO and KEGG enrichment analysis. **(A)** GO enrichment analysis results. **(B)** KEGG enrichment analysis result.

3.5. PPI Network Analysis

This study constructed a differential PPI regulatory network in the muscles of YN black pigs and HN black pigs (Figure 3). The differential PPI network was constructed using

STRING (v11.5; confidence score > 0.7) and analyzed with MCL clustering (inflation = 3.0). Key hub genes including *ELOVL6*, *PRKAG3*, and *ROCK2* were identified through betweenness centrality analysis, with their network prominence strongly supported by significant correlations with measured nutrient traits. Specifically, *ELOVL6* expression correlated with C16:0 and C18:0 fatty acids ($r = 0.72\text{--}0.75$, $p < 0.01$), while *PRKAG3* showed negative correlation with intramuscular fat content ($r = -0.69$, $p < 0.001$). A lipid metabolism cluster (*BCO1*, *ALDH1A2*, *DHRS3*, *ADH7*) enriched for GO:000629 (FDR < 0.01) further substantiated the network's relevance to observed biochemical differences.

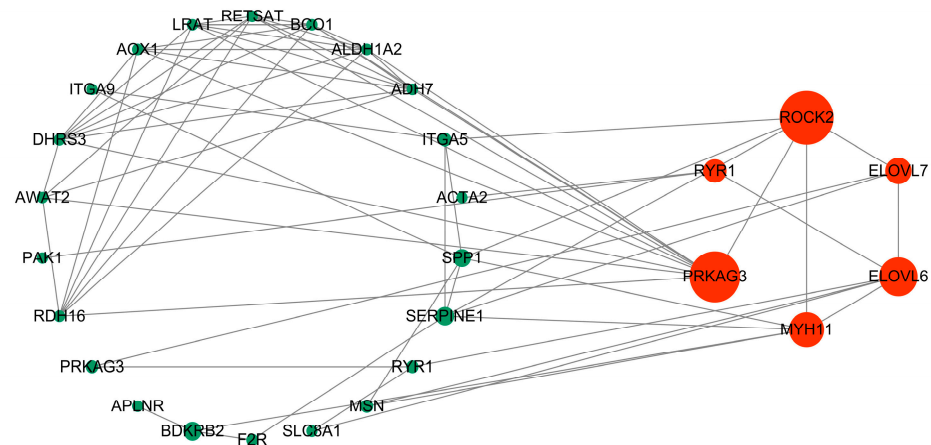


Figure 3. PPI networks of DEGs. The network was constructed using the STRING database with a confidence score threshold > 0.7. Edges (lines) represent predicted or known protein–protein interactions. Node size represents the level of betweenness centrality, indicating the importance of a protein in the network's connectivity. Red nodes indicate high betweenness centrality, while green nodes represent low betweenness centrality.

3.6. miRNA-Seq Analysis and miRNA Identification

Sequencing of small-RNA libraries from groups HN and YN groups generated 70,313,380 and 70,495,215 raw reads, respectively. After filtering, there were 69,783,924 and 68,581,130 clean reads, respectively (Table 4).

Table 4. Summary of miRNA sequencing data quality and the statistics of the transcriptome assemblies.

Sample	Raw_Reads	Clean_Reads	Q20	Q30	Q20/Q30 (%)	GC (%)
HN1	11,967,189	11,867,154	99.25	97.58	1.017	45.13
HN2	11,406,040	11,316,113	99.24	97.5	1.017	47.4
HN3	11,607,412	11,534,660	99.29	97.5	1.018	47.33
HN4	12,130,108	12,029,902	99.24	97.59	1.017	47.31
HN5	11,842,520	11,752,301	99.23	97.58	1.017	46.67
HN6	11,360,111	11,283,794	99.2	97.26	1.020	47.06
YN1	11,899,770	11,703,531	99.18	97.25	1.020	46.01
YN2	11,353,977	11,213,124	99.25	97.6	1.017	45.8
YN3	11,835,438	11,762,998	99.29	97.66	1.017	45.9
YN4	11,958,181	10,643,459	99.19	97.25	1.020	49.59
YN5	11,475,075	11,355,918	99.29	97.7	1.016	46.59
YN6	11,972,774	11,902,100	99.34	97.57	1.018	45.55

The differentially expressed miRNA (DEMs) Venn diagram was shown in Figure 4A. 125 significantly DEMs were identified in HN vs. YN, of which 64 miRNAs were up-regulated, and 61 miRNAs were down-regulated (Figure 4B). The larger fold changes in miRNA expression were ssc-miR-133b, ssc-miR-206 and miR-205.

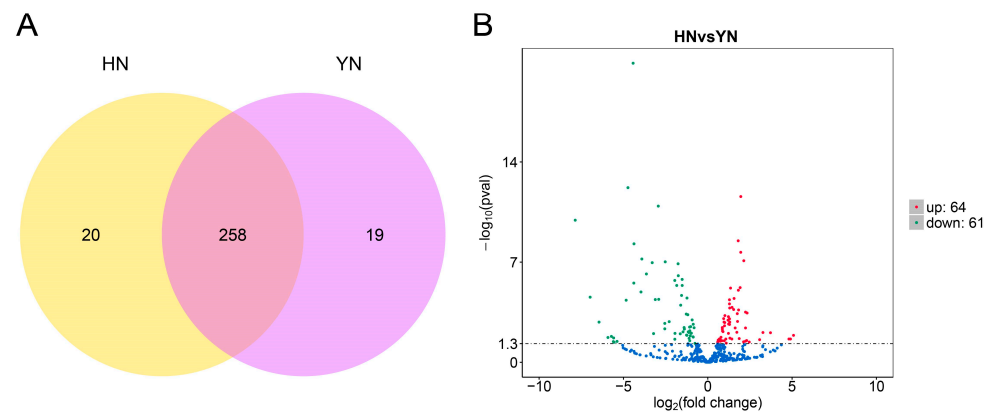


Figure 4. Identification of DEMs. (A) Venn diagram of DEMs. (B) Volcano plot of DEMs.

GO and KEGG enrichment analysis on these genes indicated that they were mainly enriched in proteolysis, phosphorylation, and polymeric cytoskeletal fiber (Figure 5A), and MAPK signaling pathway, growth hormone synthesis, secretion, and action, calcium signaling pathway (Figure 5B).

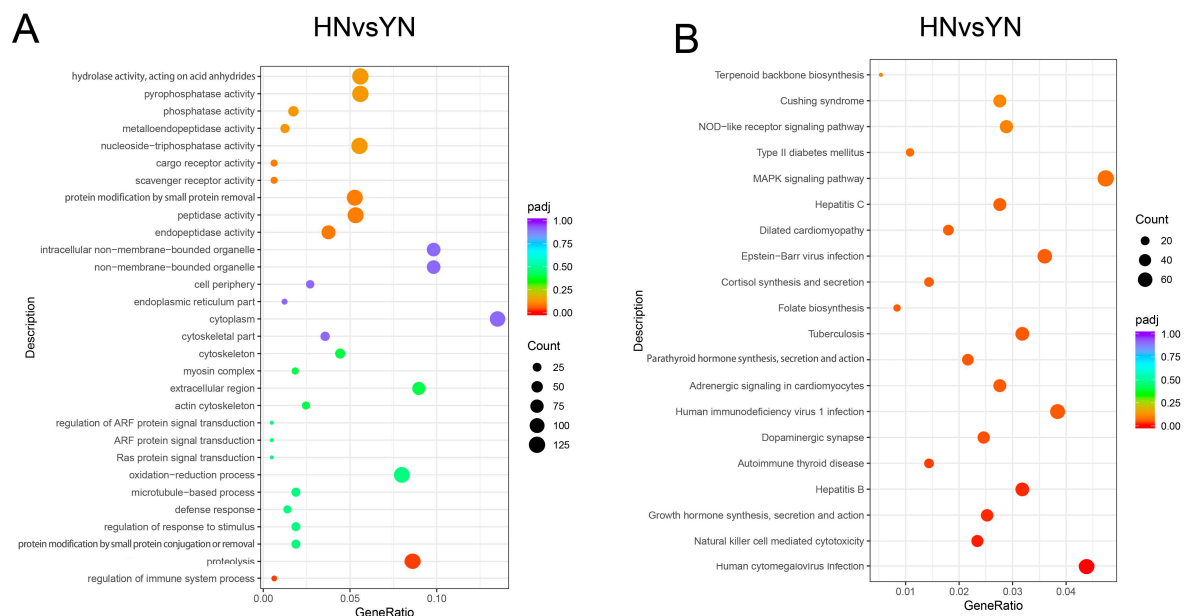


Figure 5. GO and KEGG analysis of DEMs. (A) GO enrichment analysis results. (B) KEGG enrichment analysis result.

3.7. Target Prediction of DEMs and Functional Analysis

In this study, a differential miRNA-mRNA regulatory network in the muscles of YN black pigs and HN black pigs was constructed. To enhance the reliability of the network, miRNA-mRNA interactions were predicted using an integrated approach, requiring consensus from both miRDB and TargetScan databases. Furthermore, only pairs exhibiting significant negative expression correlations (Pearson's $|r| > 0.6$, $p < 0.05$) were incorporated into the final network. As shown in Figure 6, red ellipses represent target genes, and blue triangles represent miRNAs, with the connections between them indicating the existence of these high-confidence regulatory relationships. From the regulatory network, multiple miRNAs are closely linked to target genes, forming a relatively complex regulatory network. Among them, the *XRF* gene is associated with multiple miRNAs such as ssc-miR-206, novel_223, and ssc-miR-1842. Similarly, the *MYH11* gene is also regulated by numerous miRNAs, including ssc-miR-145-5p, novel_276, ssc-miR-6782-3p, novel_261, ssc-miR-432-

5p, and ssc-miR-339-5p, among which ssc-miR-145-5p showed a strong negative correlation with *MYH11* expression ($r = -0.68$, $p < 0.01$). For the target gene *ROCK2*, it is associated with novel_243, and their expressions were inversely correlated ($r = -0.61$, $p < 0.05$); target genes such as *PRKAG3*, *ELOVL6*, *SELL*, and *NLRP5* interact with corresponding sets of miRNAs, and key pairs like *PRKAG3*-ssc-miR-365-5p also demonstrated significant negative correlations ($r = -0.65$, $p < 0.01$).

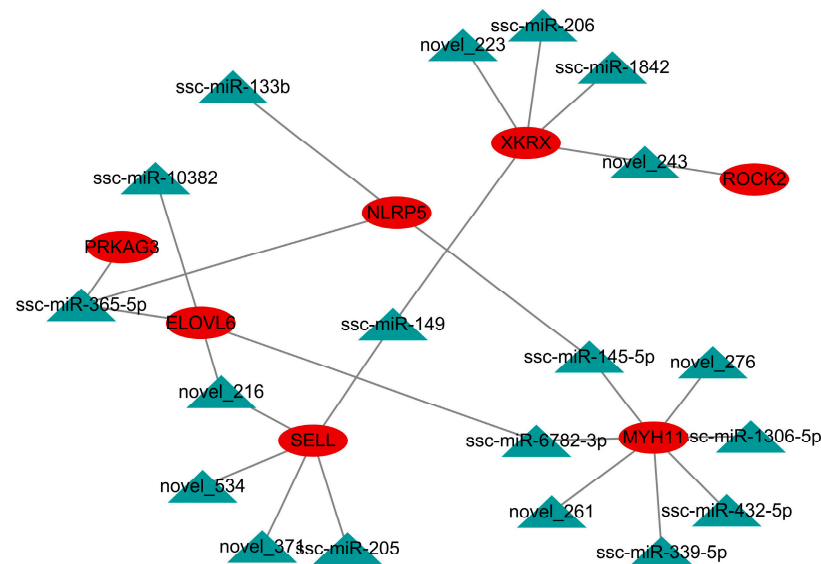


Figure 6. miRNA-mRNA regulatory network between DEMs and negatively regulated target genes. Red ovals represent target genes and blue triangles represent miRNAs.

3.8. Correlation Analysis Between Differentially Expressed Genes and Fatty Acid Content

Correlation analysis further validated the functional importance of candidate genes. As shown in Table 5, *ELOVL6* expression was strongly positively correlated with C16:0 and C18:0 content (partial correlation coefficients were 0.65 and 0.68, respectively, $p < 0.01$), consistent with its known role in fatty acid elongation.

Table 5. Correlation analysis between key genes and fatty acid content.

Fatty Acids	Gene	Partial Correlation Coefficient (r)	p-Value
C16:0	<i>ELOVL6</i>	0.65	$p = 0.003$
C16:0	<i>PRKAG3</i>	−0.52	$p = 0.015$
C18:0	<i>ELOVL6</i>	0.68	$p = 0.002$
C18:0	<i>ROCK2</i>	0.38	$p = 0.047$
C18:1n9c	<i>PRKAG3</i>	−0.57	$p = 0.008$
C18:1n9c	<i>MYH11</i>	−0.41	$p = 0.049$
C18:2n6c	<i>ELOVL6</i>	0.49	$p = 0.025$
C18:2n6c	<i>PRKAG3</i>	−0.45	$p = 0.034$

3.9. qRT-PCR Validation of mRNAs and miRNAs

To validate the RNA sequencing data, we selected a subset of differentially expressed genes, including *LDHB*, *PFKFB3*, *ACTA2*, *CD37*, *ROCK2*, and *MYH4*, as well as microRNAs ssc-miR-206, ssc-miR-486, ssc-miR-122, ssc-miR-133a, ssc-miR-378, and ssc-miR-1, for quantitative real-time PCR (qPCR) analysis (Figure 7). The qPCR results were generally consistent with the RNA-seq data, confirming the reliability and accuracy of the sequencing results. Primer sequences used for qRT-PCR validation are provided in Tables S1 and S2.

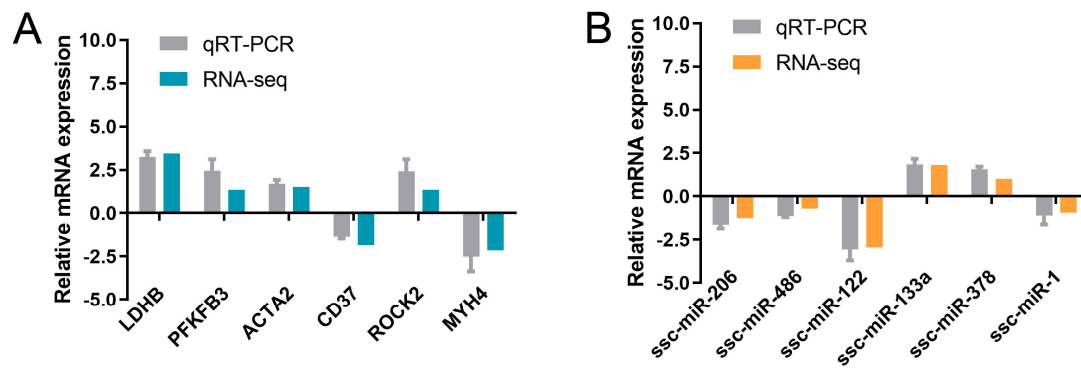


Figure 7. Verification results of DEMRNAs and DE miRNAs by qRT-PCR. (A) Relative expression values of DEMRNAs. (B) Relative expression values of DE miRNAs.

4. Discussion

As indigenous pig breeds, HN and YN black pigs have long been an integral part of local agricultural heritage in China. These breeds possess not only cultural significance but also substantial research value owing to their distinct genetic backgrounds and adaptations to local environments. The fat content of meat is closely related to meat quality, both in adipose tissue and muscle tissue, and plays a very important role in meat flavor, tenderness, water retention, and is the core of the nutritional value of meat [20]. Among saturated fatty acids, palmitic acid and stearic acid are particularly noteworthy. Palmitic acid serves as an essential fatty acid in humans and promotes nutrient absorption in infants and young children [21], whereas stearic acid has been shown to mitigate cholestasis-induced liver injury [22].

From a metabolic perspective, HN black pigs demonstrate significantly higher contents of C18:1n7c and C18:2n6c, indicating potentially enhanced activity of Δ^9 -desaturase (*SCD1*) and fatty acid desaturase (*FADS*) genes [23]. This interpretation is supported by the significantly lower C16:0/C16:1 and C18:0/C18:1 ratios in HN compared to YN black pigs, consistent with the role of *SCD1* in converting saturated to monounsaturated fatty acids [24]. The distinct fatty acid profile of HN black pigs may reflect improved lipid metabolic characteristics, as elevated oleic acid is known to activate PPAR γ signaling pathways that regulate lipid metabolism [25]. Furthermore, HN black pigs show superior capacity in accumulating n-3 series fatty acids (e.g., α -linolenic acid), potentially attributed to breed-specific polymorphisms in elongase (*ELOVL*) and desaturase (*FADS1/2*) genes, or gut microbiota-mediated short-chain fatty acid metabolism [26,27]. The pronounced divergence in C18:3n3 content between breeds suggests adaptation to local nutritional environments through specialized lipid metabolic networks. Comparative genomic studies have identified selection signatures in key lipogenic genes such as fatty acid synthase (*FASN*) and acetyl-CoA carboxylase (*ACACA*) among Chinese indigenous pig breeds [28], supporting these role in shaping fatty acid composition. Additionally, the breed-specific MUFA/PUFA ratio in HN black pigs may contribute to improved oxidative stability and meat preservation properties.

Amino acids significantly influence muscle development, flavor characteristics, and nutritional quality in meat products [29]. Our comparison revealed that HN black pigs contain significantly higher muscular levels of specific amino acids, including aspartic acid, glutamic acid, valine, leucine, and histidine, compared to YN black pigs. Notably, valine and leucine are classified as branched-chain amino acids (BCAAs), which support protein synthesis and energy metabolism [30], and contribute to muscle development in livestock [31]. Among non-essential amino acids, aspartic acid and glutamic acid are key contributors to the umami flavor profile, a critical determinant of consumer preference [32].

The elevated levels of these amino acids in HN black pigs suggest a more pronounced umami character in their meat. Histidine, an essential amino acid, serves as a building block for muscle proteins and participates in various metabolic processes [33]. It also contributes to antioxidant defense systems, which may influence meat quality during storage and processing [34]. The higher histidine levels in HN black pigs could therefore enhance oxidative stability in muscle tissues, thereby influencing overall sensory quality.

To elucidate the molecular mechanisms underlying meat quality differences between HN and YN black pigs, we conducted transcriptome and PPI analyses. These analyses identified *ELOVL6*, *ELOVL7*, and *RYR1* as core regulatory genes. Specifically, *ELOVL6* expression was significantly elevated in HN compared to YN black pigs. The *ELOVL6* gene encodes a key enzyme in fatty acid elongation and has been established as a major quantitative trait locus (QTL) on chromosome 8 influencing palmitic and palmitoleic acid content in porcine muscle and adipose tissue [35]. This gene is particularly important in the context of meat quality traits in pigs. Previous studies indicate that enhanced *ELOVL6* activity promotes long-chain fatty acid synthesis, which can influence marbling and tenderness [36]. As suggested by studies on *ELOVL6* knockout mice, changes in *ELOVL6* expression could impact insulin sensitivity, potentially influencing overall pig health and growth efficiency [37]. Furthermore, reports similar to this study reported that the *ELOVL6* gene expression of local Alentejano (AL) and Bísaro (BI) pigs in Portugal was significantly higher than that of lean breeds [38]. The higher expression of *ELOVL6* gene in HN black pigs in our study suggests an enhanced capacity for fatty acid elongation, potentially contributing to the distinct fatty acid composition and meat quality characteristics of this breed.

PRKAG3 was identified as a core gene distinguishing HN and YN black pigs. This gene encodes a regulatory subunit of AMP-activated protein kinase (AMPK) and plays a critical role in skeletal muscle energy metabolism and homeostasis [39,40]. The expression level of *PRKAG3* has been associated with muscle development and meat quality traits across pig breeds [41]. In this study, HN black pigs recognized for their favorable meat quality and growth performance, which exhibited higher *PRKAG3* expression than YN black pigs. Elevated *PRKAG3* expression may promote oxidative metabolism and glycogen storage in muscle, potentially influencing fiber type composition and intramuscular fat deposition [42]. These metabolic characteristics are consistent with improved marbling, tenderness, and pH stability—key attributes of meat quality [43]. Additionally, the metabolic advantages conferred by elevated *PRKAG3* levels may lead to better meat color and pH stability, both of which are critical for consumer acceptance and shelf-life of pork products.

The *ROCK2* gene encodes a critical regulator of cellular signaling pathways involved in muscle contraction, cell growth, and cytoskeletal dynamics [44]. Its role in muscle physiology makes it a significant focus in studies related to muscle development, meat quality, and overall growth performance in livestock [45]. In our study, YN black pigs exhibited higher *ROCK2* expression compared to HN black pigs, suggesting potentially enhanced muscle fiber development. This observation aligns with previous findings that *ROCK2* promotes myoblast proliferation and differentiation, the key processes in muscle growth [46]. Beyond its role in myogenesis, *ROCK2* expression may influence metabolic processes in muscle tissue [47]. The gene's documented involvement in regulating muscle contraction and relaxation further suggests that its differential expression could affect muscle fiber characteristics relevant to meat texture [48,49]. As tenderness is a crucial attribute in pork quality, HN black pigs may have a competitive edge in meat markets. Additionally, given the established role of ROCK signaling in adipogenesis, the observed variation in *ROCK2* expression between breeds may contribute to differences in intramuscular fat deposition and marbling formation [50].

The *MYH11* gene encodes a myosin heavy chain predominantly expressed in smooth muscle [51]. As fundamental contractile proteins, myosins play a key role in muscle contraction and performance [52]. In the present study, HN black pigs exhibited higher *MYH11* expression levels than YN black pigs. Elevated *MYH11* expression has been associated with improved muscle structure and contractile properties, which may influence meat texture and tenderness [53,54]. These findings suggest that differential *MYH11* expression between breeds could contribute to variations in muscle characteristics and meat quality attributes.

miRNAs are small, non-coding RNAs that post-transcriptionally regulate gene expression and play important roles in muscle development and growth [55,56]. Based on RNA-seq data, we identified several key miRNAs associated with meat quality traits, including ssc-miR-133b, ssc-miR-206 and miR-205. Among these, ssc-miR-133b is a well-characterized regulator of muscle differentiation and proliferation [57]. In the present study, ssc-miR-133b expression was significantly lower in HN black pigs than in YN black pigs. It regulates key myogenic factors and pathways, thereby influencing muscle development and growth. HN black pigs are recognized for their adaptability to local environments, but they possess traits that may limit their muscle development compared to other breeds [1]. In contrast, YN black pigs are known for higher market value and superior meat quality, which may correlate with their higher expression of muscle-related miRNAs, including ssc-miR-133b [58]. The significant reduction in ssc-miR-133b in HN black pigs compared to YN black pigs suggests that lower levels of this miRNA could contribute to differences in muscle growth and development. Lower levels of ssc-miR-133b may impair muscle proliferation and differentiation, leading to reduced muscle fiber size and density [59]. In contrast, the higher expression observed in YN black pigs likely facilitates more robust muscle development and better meat yield.

In addition, ssc-miR-206 was significantly differentially expressed between HN and YN black pigs. ssc-miR-206 plays an important role in muscle biology, where it promotes myogenesis and regeneration primarily through targeting inhibitors of muscle differentiation [60]. We observed significant down-regulation of ssc-miR-206 in HN black pigs compared to YN black pigs. As ssc-miR-206 is known to target transcriptional repressors that inhibit myoblast differentiation, its reduced expression in HN black pigs may be associated with altered regulation of muscle development pathways [61]. Therefore, the lower levels of ssc-miR-206 in HN black pigs may lead to increased expression of its target inhibitory proteins, thereby reducing myoblast proliferation and differentiation. This altered regulatory pattern could contribute to impaired muscle growth, potentially affecting overall muscle mass and meat quality in this breed. Meanwhile, miR-205 has been established as a regulator of muscle differentiation through its modulation of pathways controlling myoblast proliferation and maturation [62]. Given the documented correlation between miR-205 expression and muscle fiber development in pigs [63], its reduced expression may be associated with differences in muscle growth characteristics between the two breeds. The regulatory influence of miR-205 is further supported by its ability to modulate multiple target genes within key muscle signaling pathways [64].

The core genetic markers identified in our study are consistently implicated in meat quality regulation across diverse pig breeds, underscoring their fundamental biological roles. *ELOVL6* variants and expression are linked to fatty acid profiles in breeds from Alentejano to Duroc [38,65], while the *PRKAG3* RN- allele profoundly affects glycogen and pH in Hampshire pigs [39,66]. Similarly, *ROCK2* and *MYH11*, key to muscle structure, show differential expression in breeds with divergent muscularity [49,54], and myogenic miRNAs like ssc-miR-206 and ssc-miR-133b are established regulators of growth in Landrace and Meishan pigs [60,67]. This cross-breed conservation highlights that we have identified a central regulatory network.

The unique expression patterns and interactions we report for HN and YN pigs thus provide novel, breed-specific insights into this common genetic framework.

5. Conclusions

This study establishes an integrated molecular framework underlying the distinct meat quality traits between HN and YN black pigs. The superior nutrient profile of HN pigs was strongly associated with the coordinated regulation of key genes (*ELOVL6*, *PRKAG3*, *ROCK2*, *MYH11*) involved in lipid and energy metabolism, further modulated by a high-confidence miRNA-mRNA regulatory network. While these findings demonstrate robust correlations rather than established causation, they provide a coherent biological narrative connecting transcriptional regulation with nutrient composition, offering valuable insights for future molecular breeding strategies.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture15242532/s1>, Table S1: Primer sequences of DEmRNAs used for qRT-PCR validation, Table S2: Primer sequences of DEmiRNAs used for qRT-PCR validation.

Author Contributions: Conceptualization, L.W., K.D. and J.H.; methodology, C.L., R.W., B.W., W.L., C.Z., J.S., Y.X. and Y.B.; software, Y.W.; validation, R.W. and Y.X.; formal analysis, L.Z., X.L., J.S., Y.X., Y.W., H.L. and Y.B.; investigation, C.L., R.W., B.W. and W.L.; resources, L.Z. and X.L.; data curation, Y.W. and H.L.; writing—original draft preparation, Y.W.; writing—review and editing, Y.W.; visualization, H.S.; supervision, W.Z.; project administration, J.H., K.D. and L.W.; funding acquisition, J.H., K.D. and L.W. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by National Key R & D Program of China (2021YFD1301201); National Natural Science Foundation of China (32172862, 32473037, 32302719); Science and Technology Innovative Research Team in Higher Educational Institutions of Henan Province (24IRT-STHN035); the joint fund of science and technology research and development plan in Henan province (225200810044); Science and Technology Project of Henan Province (232102110008, 242102111009, 2023HYTP026); the joint fund of science and technology research and development plan in Henan province (225200810044).

Institutional Review Board Statement: All animal studies were conducted in accordance with the experimental practices and standards of the Animal Welfare and Research Ethics Committee of Henan Institute of Science and Technology (No. 2024667) issued on 7 March 2022.

Data Availability Statement: Data are available on request to the authors.

Conflicts of Interest: The authors declare no competing interests.

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