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Comparative transcriptomic analysis reveals beneficial effect of dietary mulberry leaves on the muscle quality of finishing pigs

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Abstract

Background: The aim of this study was to investigate the effect of dietary mulberry leaves on the transcriptome profiles of finishing pigs. RNA-Seq was used to identify differentially expressed genes (DEGs) in the longissimus dorsi of 56 pigs fed either a traditional diet or diets supplemented with 3%, 6% or 9% mulberry leaf powder, and both gene ontology (GO) function and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analyses were performed. Furthermore, protein–protein interaction (PPI) network and the subnet module analysis were used to identify genes with beneficial potential, and quantitative real-time polymerase chain reaction (qRT-PCR) was used to validate the expression patterns revealed by RNA-Seq.

Results: Pigs fed with the 6% mulberry diet exhibited greater average daily gain, lower water loss and lower shear force than the control group and yielded 531 DEGs, including 271 and 260 upregulated and downregulated genes, respectively. Function analysis revealed that the DEGs were significantly enriched in functions related to muscle growth and development. Furthermore, several genes (i.e. *ACOT4*, *ECHS1*, *HACD1*, *NPR1*, *ADCY2*, *MGLL* and *IRS1*) were enriched in a KEGG pathway that was associated with fatty acid metabolism, and in the PPI subnet module, four of eight node genes, namely *TNNC1*, *MYL3*, *TCAP* and *TNNT1*, were associated with muscle formation and development. The upregulation of these genes, including *TNNC1*, *TNNT1* and *MYL3*, was confirmed by qRT-PCR.

Conclusions: Dietary mulberry leaves (6%) may improve the muscle quality of pigs by modulating the expression of several key genes, such as *TNNC1*, *MYL3* and *TNNT1*.

KEYWORDS
differentially expressed gene, mulberry, pig

1 | BACKGROUND

Along with improving quality of life, one of the main concerns in meat production is to improve meat quality. For pork production, the index used to evaluate meat quality has typically included various characteristics, including flesh colour, amount of intermuscular fat, tenderness and extreme pH. However, researchers have now begun to focus on improving additional measures of pork quality (e.g. free fatty acid concentrations and oxidative stability) by supplementing pig feed with various additives, including selenium, lysine, oregano oil, N-carbamylglutamate and chicorytosan (Calvo et al., 2017; Schwarz et al., 2017).

The leaves of mulberry trees, which are widely distributed and abundant throughout China, are traditionally used for rearing silkworm (Acharya, Ghosh, & Kundu, 2008). In China, mulberry trees are distributed widely throughout the country, and mulberry resources are abundant (Liu et al., 2016). However, mulberry leaves have not been fully utilized for other applications, especially after late autumn. As a traditional Chinese herbal medicine, mulberry leaves are rich in nutrients and active substances (Fan & Xu, 2017; Zeng et al., 2016). The abundant resources and exceptional nutritional value of mulberry leaves have prompted researchers to investigate the use of mulberry leaves as a foodstuff for the husbandry of both livestock and poultry (Li et al., 2017; Ly & Samkol, 2014). In fact, previous studies have demonstrated that mulberry leaves can significantly improve animal production performance, feed conversion efficiency and meat quality (Eo & Lim, 2016), and in animal husbandry, mulberry leaves can be not used as both a green feed and a protein feed. Researchers have proposed that the addition of mulberry leaves to poultry diets could significantly increase body metabolism, body growth and production performance and significantly reduce heavy metal accumulation and ammonia emissions (Fan et al., 2017; Kobayashi, Miyazawa, & Kamei, 2010), and Martínez, Motta, Cervera, and Pla (2005) reported that supplementing rabbit diets with rabbit leaves resulted in leaner carcasses that had leaner meat and increased moisture levels. However, the effects of dietary mulberry leaves on pig production are yet to be fully investigated. Even though research has clearly demonstrated that mulberry leaves can improve meat quality and reduce food cost (Tingjuan, Mengzhou, & Lianghong, 2017), the evaluation of dietary supplementation in pigs has primarily focused on the physiological and biochemical analysis of pork quality, and the metabolic pathways and gene regulation associated with improvements in the quality of mulberry-fed pork remain unknown.

Accordingly, the aim of the present study was to explore the molecular mechanisms associated with dietary mulberry supplementation by transcriptome profiling in pigs. To understand the genetic factors associated with meat quality improvement associated with mulberry leaves, differentially expressed genes (DEGs) and potential gene networks affecting quality improvement in pigs were explored between RNA profiling of pigs fed with mulberry leaves as compared with pigs fed with conventional feed.

2 | MATERIAL AND METHODS

2.1 | Experimental animals and sample collection

The conventional feed and mulberry leaf powder were obtained from Shanxi ankang yangsheng Biological Feed Technology Co., Ltd, and the composition and major bioactive ingredients of the mulberry leaf powder are summarized in Table S1. A total of 56 35-day-old, healthy Landrace-Yorkshire hybrid pigs (31.18 ± 4.57 kg) were divided into four groups, with 14 pigs (seven female and seven male) assigned to each of four treatment groups, and the four groups of pigs were housed in the same pen and railed off. Pigs in group I were fed a basal diet, whereas those in groups II, III and IV were fed basal diets that were supplemented with either 3%, 6% or 9% mulberry leaf powder, respectively, and all the diets were maintained for 102 days. The composition and nutritional content of the diets are summarized in Table S2. There was no significant difference in the starting weights of the treatments groups ($p > 0.05$), and the four groups were housed in the same pen, although separated using rails, and allowed to drink freely throughout the study. The daily feed intake of each group was recorded daily, along with daily weather changes, and both the excrement and health status of the pigs were observed at all times. The optimal level of mulberry leaf powder supplementation was determined by comparing the daily gain, average daily feed intake, feed meat ratio, meat colour, pH at 45 min and 24 hr post-mortem, water loss, cooked meat percentage, drip loss, shear force and muscle tenderness of the treatment groups.

The experimental protocol used for the present study was approved by the Animal Care and Use Committee of Gansu Agricultural University (Lanzhou, China).

After 102 days, the pigs were anaesthetized, using CO_2, and slaughtered at Ankang Minrong slaughtering house. Briefly, when the pigs lost consciousness, fell to the ground, and remained completely motionless for ~ 1 min, they were hung upside down for bloodletting. After slaughter, the longissimus dorsi between the third thoracic and fourth lumbar vertebrae were selected to assess meat quality and flavour to obtain material for molecular analysis. All the samples were collected rapidly after slaughter and then stored in liquid nitrogen.

2.2 | Determination of muscle nutrients

The water loss rate, crude protein, fat and ash levels and pH of the muscle samples were measured according to the Association of Official Analytical Chemists (AOCC) (Bader & Hogue, 2003). Crude fat was measured using the Soxhlet extraction method, and crude ash was estimated using a 550°C ashing furnace.

2.3 | RNA extraction, library construction and sequencing

Total RNA was extracted from the longissimus dorsi tissue samples (50–100 mg) of 14 pigs that had been fed the basal diet and 14 pigs that had been fed the basal diet supplemented with 6% mulberry leaf powder. For each sample, RNA was extracted using 1-ml TRIZOL, chilled on ice for 5 min, combined with 200-μl chloroform and then centrifuged at 12,000*g* for 15 min. Each supernatant was subsequently transferred to a sterile tube, combined with an equal amount of isopropanol, incubated at − 20°C and centrifuged at 12,000*g* for 15 min. The resulting sediment was washed using 1-ml 75% ethanol, centrifuged at 12,000*g* for 5 min, air dried for 5–10 min and suspended in 20-μl diethyl pyrocarbonate water. The quality of the RNA was measured using an Infinite M100 PROBio analyzer (TECAN, Switzerland), and all the experiments were repeated three times.

Poly-dT oligonucleotides were used to reverse-transcribe RNA into cDNA, and after end repair, the cDNA was subject to A-tailing and adaptor ligation, followed by cDNA purification and enrichment. Sequencing was performed using an IlluminaHiSeq2000 with 100-bp paired read sequencing.

2.4 | Mapping the reads to reference genome

Before mapping, a quality check was performed using Trimmomatic (Bolger, Lohse, & Usadel, 2014), and the raw sequences were processed to (a) remove linker sequences and reads resulting from linker self-ligation; (b) remove low-mass (<20) bases at the 3′-end of sequences and eliminate remaining sequences with mass values of < 10; (c) remove reads containing > 10% undetermined bases; and (d) discard adapters and mass-pruned sequences of < 20 bp in length. Finally, clean data from two samples were subject to further analysis.

The filtered reads were mapped to the Sus_Scrofa genome and annotated using TopHat (v2.1.0; http://ccb.jhu.edu/software/tophat) (Trapnell, Pachter, & Salzberg, 2009). Up to four mismatched bases were allowed, and all other parameters were set as default.

2.5 | Transcript assembly, quantification and DEGs analysis

Using the cufflinks tool (v2.2.1) (Trapnell et al., 2010), transcript reassembly was performed on six samples using the Sus_scrofa genome annotation file (Ensembl, v 91) as a reference (Aken et al., 2016), and the resulting multiple gtf files were fused into a more comprehensive transcript. The annotation results were then used to quantify individual sample gene and transcript expression levels using cuffquant and cuffnorm, and both read count and fragments per kilobase of transcript per million mapped reads quantitative results were obtained. Finally, DEG analysis was performed using cuffdiff and a threshold of |log2FC|>0.585 and $p < 0.05$.

2.6 | Gene ontology (GO) and pathway analyses

Cluster profiler (v6.8) was used to perform gene ontology (GO) function and KEGG pathway enrichment analyses of genes that corresponded to significant differential transcripts (Yu, Wang, & Han, 2012). Hypergeometric test p-values of < 0.05 were considered to indicate significant enrichment. The genes enriched in the biological process, functional function, functional concentration in the GO function and KEGG pathway enrichment analyses were obtained.

2.7 | Protein–protein interaction (PPI) network

The PPI network of the significant differential transcript counterparts was analysed using the STRING online tool (Szklarczyk et al., 2015), with a required confidence (combined score) threshold of > 0.4.

Cytoscape tools were used to build a protein interaction network, analyse network topology and identify important nodes on the basis of network connectivity (Shannon et al., 2003). The importance of nodes in the network was assessed by measuring degree centrality, betweenness centrality and closeness centrality.

In the living body, essential genes with higher degrees of centrality, or their translation products, support the basic activities of life and, therefore, appear in the entire biological network at a high frequency, as do many genetic disease-related genes (Yoon, Blumer, & Lee, 2006). In the present study, the connectivity of a node was defined as the number of direct connections to other nodes, and degree of connectivity indicated the importance of each node in the network. The node with the highest connectivity was recognized as the hub node.

Betweenness centrality was used to demonstrate the role of genes in maintaining network tightness (Cukierski & Foran, 2008). Media centeredness indicated the role played by a certain node in connection with other nodes. The node would play better betweenness centrality when the larger value was calculated.

Closeness centrality was as an index of the score of the node in the network (Du et al., 2015). The intimacy coefficient was also called tightness, and these values indicated the distance of genes to the centre of the network. The cytoscape plug-in CytoNCA (parameter settings: network with no weight) (Tang, Li, & Wang, 2015) was used for the three central methods of the computational network, and node scores indicated the importance of genes in the network.

2.8 | Module analysis

In PPI networks, proteins with similar functions tend to cluster together, and network node distances are related to node functions. Therefore, investigating protein complexes or functional clustering modules in PPI networks can provide insight into the role of proteins with unknown functions. Accordingly, the subnet module of the PPI relational network was analysed using MCODE (Bader & Hogue, 2003).

2.9 | Validation of gene expression

QRT-PCR was used to validate the relative expression patterns obtained *via* RNA-Seq. More specifically, single-stranded cDNA was synthesized from 0.5 μg total RNA using 5 × prime Script RT Master MIX (TAKARA Biotechnology, Dalian, China) following the manufacturer's instructions, and quantitative PCR analysis was performed for *TNNC1*, *MYL3*, *TNNT1*, *TCAP*, *ADCY2* and *HACD1* using SYBR premix EX taq, an ABI 7,500 Real-Time PCR System (7900HT FAST, Applied Biosystems), and the following primers: TNNC1-sF (GATGACATCTACAAGGCTGCG), TNNC1-sR (GCACGAAGATGTCAAAGGCT), MYL3-sF (AGGACTTTGTGGAAGGGCTG), MYL3-sR (TCTTGCCCAGCCATCAACTT), TNNT1-sF (CATCCGCGGTTTAGGAATCTTT), TNNT1-sR (CCCCCTCTGGGATCTTCGG), TCAP-sF (CGTGAGACCTACCACCAACAG), TCAP-sR (CCACTT

TABLE 1 Effect of dietary mulberry powder on the growth performance, production performance and meat quality of finishing pigs

		Group I	Group II	Group III	Group IV
	Item	*N* = 16	*N* = 16	*N* = 16	*N* = 16
Growth performance	Initial weight (kg)	31.43 ± 3.17	31.29 ± 2.26	31.07 ± 2.08	30.93 ± 2.35
	Final weight (kg)	118.29 ± 4.36^{b}	116.21 ± 3.12^{b}	127.36 ± 4.87^{a}	118.69 ± 4.49^{b}
	Average daily gain (g)	852 ± 38.57^{b}	833 ± 36.24^{b}	944 ± 43.16^{a}	927 ± 39.05^{a}
	Average daily feed intake (kg)	2.49 ± 0.26	2.26 ± 0.15	2.57 ± 0.39	2.39 ± 0.18
	Feed:meat ratio	2.92 ± 0.21	2.72 ± 0.14	2.72 ± 0.17	2.99 ± 0.31
Production performance	Half carcass weight (kg)	94.83 ± 2.37	89.33 ± 2.08	94.20 ± 1.18	91.43 ± 4.54
	Slaughter rate (%)	76.68 ± 0.36	75.49 ± 0.43	75.97 ± 0.95	73.94 ± 3.58
	Average backfat thickness (mm)	9.91 ± 1.64	9.38 ± 1.34	9.74 ± 1.58	9.83 ± 0.70
	Loin eye area (cm^2)	45.38 ± 7.87	40.56 ± 9.83	50.38 ± 0.49	43.03 ± 7.68
Meat quality	Cooked meat percentage (%)	64.77 ± 3.64	61.21 ± 0.27	62.40 ± 1.78	64.52 ± 1.27
	Water loss rate (%)	2.46 ± 1.73^{a}	2.43 ± 1.81^{ab}	1.66 ± 0.56^{b}	1.56 ± 0.31^{b}
	Drip loss (%)	19.40 ± 0.82	19.33 ± 1.31	19.64 ± 6.26	19.13 ± 9.39
	Shear force (*N*)	70.30 ± 29.91^{a}	56.74 ± 23.40^{b}	47.28 ± 19.44^{b}	53.93 ± 29.76^{b}
	Meat colour	3.53 ± 0.97	4.10 ± 1.20	3.70 ± 1.65	3.97 ± 0.91
	pH (45 min)	5.96 ± 0.21	6.01 ± 0.10	5.96 ± 0.49	5.83 ± 0.57
	pH (24 hr)	5.44 ± 0.26	5.82 ± 0.22	5.68 ± 0.09	5.64 ± 0.18
	Water (%)	71.52 ± 0.21	71.64 ± 0.33	72.13 ± 0.80	72.42 ± 1.23
	Crude protein (%)	19.05 ± 0.70	20.23 ± 0.32	19.59 ± 2.79	18.42 ± 0.68
	Crude ash (%)	27.20 ± 0.30	26.92 ± 0.26	26.62 ± 0.78	26.19 ± 1.01
	Crude fat (%)	2.23 ± 0.34	2.30 ± 0.12	2.28 ± 0.79	1.80 ± 0.77

Note: The data were presented as mean ± standard deviation (*SD*). Peer data with no shoulder or shoulder note with the same letter indicate no significant difference ($p > 0.05$), adjacent letters indicate significant difference ($p < 0.05$) and separated letters indicate extremely significant difference ($p < 0.01$).

TABLE 2 Summary of reads, bases and the transcript-annotated bases per pig

Sample	Raw reads	Clean reads	Effective rate (%)	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
C1	27,608,848	27,296,417	98.87	0.02	96.49	91.37	54.61
C2	21,721,642	21,533,989	99.14	0.02	96.47	91.23	54.27
C3	30,667,844	30,370,701	99.03	0.02	96.86	92.13	55.03
M1	23,767,135	23,530,919	99.01	0.02	96.47	91.32	54.84
M2	27,943,959	27,561,073	98.63	0.02	96.93	92.2	53.44
M3	27,597,924	27,202,868	98.57	0.02	96.8	91.7	54.34

Note: Abbreviations: GC content, percentage of total bases comprised of G/C bases; Q20, percentage of Phred values> 20; Q30, percentage of Phred values> 30. C1, C2 and C3 are control group samples, whereas M1, M2 and M3 are samples from the mulberry powder group.

TGGCAGGGGTGAAG), ADCY2-sF (AGCATGACCACAGAGAACGG), ADCY2-sR (GGCAGTGGCTCGGTATTCTT), HACD1-sF (GGTGT GGCTCGTTACTCACA), and HACD1-sR (AAGTACGGCAAG TGGTCGAG. The mRNA levels were normalized to the levels of *GAPDH* using the following primers: GAPDH-sF (TCGGA GTGAACGGATTTGGC) and GAPDH-sR (TGACAAGCTTCCCGT TCTCC). The amplification was performed in triplicate, and the relative expression levels of the genes were calculated using the $2^{-\Delta\Delta CT}$ method.

2.10 | Statistic analysis

The growth performance, production performance and meat quality of the four treatment groups were compared using SPSS 22.0,

and all results were calculated as mean ± standard deviation (*SD*) values. The significance of differences between group pairs was calculated using Student's *t* test, and the significance of differences between three groups was analysed using one-way analysis of variance (ANOVA), followed by post hoc least significant difference tests. *p*-values of < 0.05 indicated statistical significance. Graphpad prism 5 (Graphpad Software, San Diego, CA, USA) was used to generate graphs. The threshold was defined as $p < 0.05$.

3 | RESULTS

3.1 | Effect of mulberry powder

The beneficial effect of dietary mulberry leaves on pig performance, including growth performance, production performance and meat quality is shown in Table 1. Pigs fed the 6% mulberry diet exhibited greater average daily gain, which was selected for the following bioinformatics

TABLE 3 Clean reads mapped to reference genomes

		C1	C2	C3	M1	M2	M3
Left reads	Input	27,296,417	21,533,989	30,370,701	23,530,919	27,561,073	27,202,868
	Mapped	24,293,560	19,025,149	26,769,555	20,734,723	24,450,431	24,111,637
	Mapped rate (%)	89.00	88.35	88.14	88.12	88.71	88.64
	Uniquely mapped	22,770,049	17,873,293	24,985,873	19,491,368	23,007,094	22,621,345
	Uniquely mapped rate (%)	83.42	83.00	82.27	82.83	83.48	83.16
Right reads	Input	27,296,417	21,533,989	30,370,701	23,530,919	27,561,073	27,202,868
	Mapped	23,862,418	18,607,271	26,501,605	20,324,256	24,301,235	23,621,061
	Mapped rate (%)	87.42	86.41	87.26	86.37	88.17	86.83
	Uniquely mapped	22,359,507	17,474,538	24,731,222	19,100,779	22,865,598	22,149,401
	Uniquely mapped rate (%)	81.91	81.15	81.43	81.17	82.96	81.42
Overall read mapping rate (%)		88.20	87.40	87.70	87.20	88.40	87.70

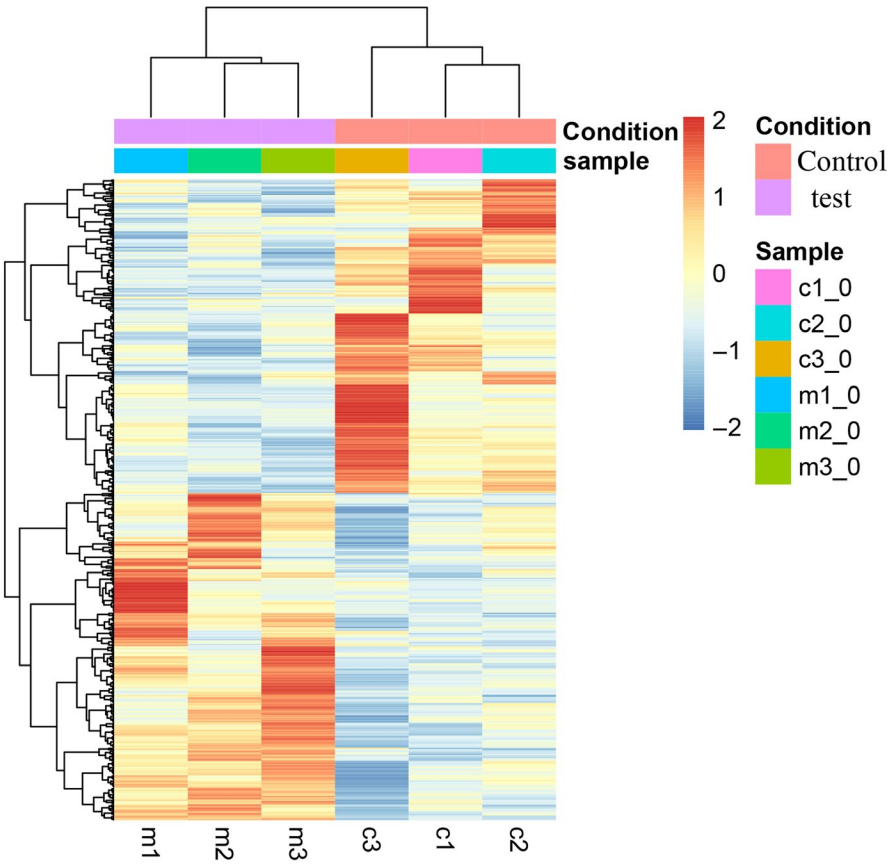

FIGURE 1 Two-dimensional clustering heat maps of differentially expressed transcript. Columns represent different samples, whereas rows represent differentially expressed genes, with red indicating high expression and blue indicating low expression

analysis, and the pigs fed 6% and 9% mulberry diets exhibited lower water loss rates and shear force values than the control group. However, diet failed to significantly affect feed:meat ratio, half carcass weight, slaughter rate, average backfat thickness or loin eye area.

3.2 | Transcriptome mapping

The summary of reads and genes mapped to the Sus_Scrofa transcriptome is shown in Tables 2 and 3. A total of 157,495,967 clean reads were obtained from the 159,307,352 raw reads (C1: 27,608,848; C2: 21,721,642; M1: 23,767,135; C3: 30,667,844; M3: 27,597,924; M2: 27,943,959). Of the total clean reads from left, 89.00%, 88.35%, 88.14%, 88.12%, 88.71% and 88.64% of reads from the C1, C2, C3, M1, M2 and M3 samples, respectively, were aligned to the Sus_scrofa genome. Based on right reads, 87.42%, 86.41%, 87.26%, 86.37%, 88.17% and 86.83% of reads from the C1, C2, C3, M1, M2 and M3 samples, respectively, were aligned to the Sus_scrofa genome.

3.3 | Differentially expressed genes (DEGs)

Of 531 significant DEGs (under the threshold of |log2FC|>0.585 and $p < 0.001$), 271 were upregulated and 260 were downregulated (Figure 1).

3.4 | Function variation

In the GO analysis, DEGs were significantly enriched in functions related to muscle growth and development, and a total of 27 genes (corresponding to 30 transcripts) were associated with the process (Figure 2, Table S1). Meanwhile, the KEGG pathway enrichment analysis revealed that DEGs enriched 13 pathways, including the apelin, cGMP-PKG and notch signalling pathways. Several genes, including *ACOT4*, *ECHS1*, *HACD1*, *NPR1*, *ADCY2*, *MGLL* and *IRS1*, were also enriched in KEGG pathways associated with fatty acid metabolism (i.e. ssc00062: fatty acid elongation, ssc04923: regulation of lipolysis in adipocytes; Figure 2d).

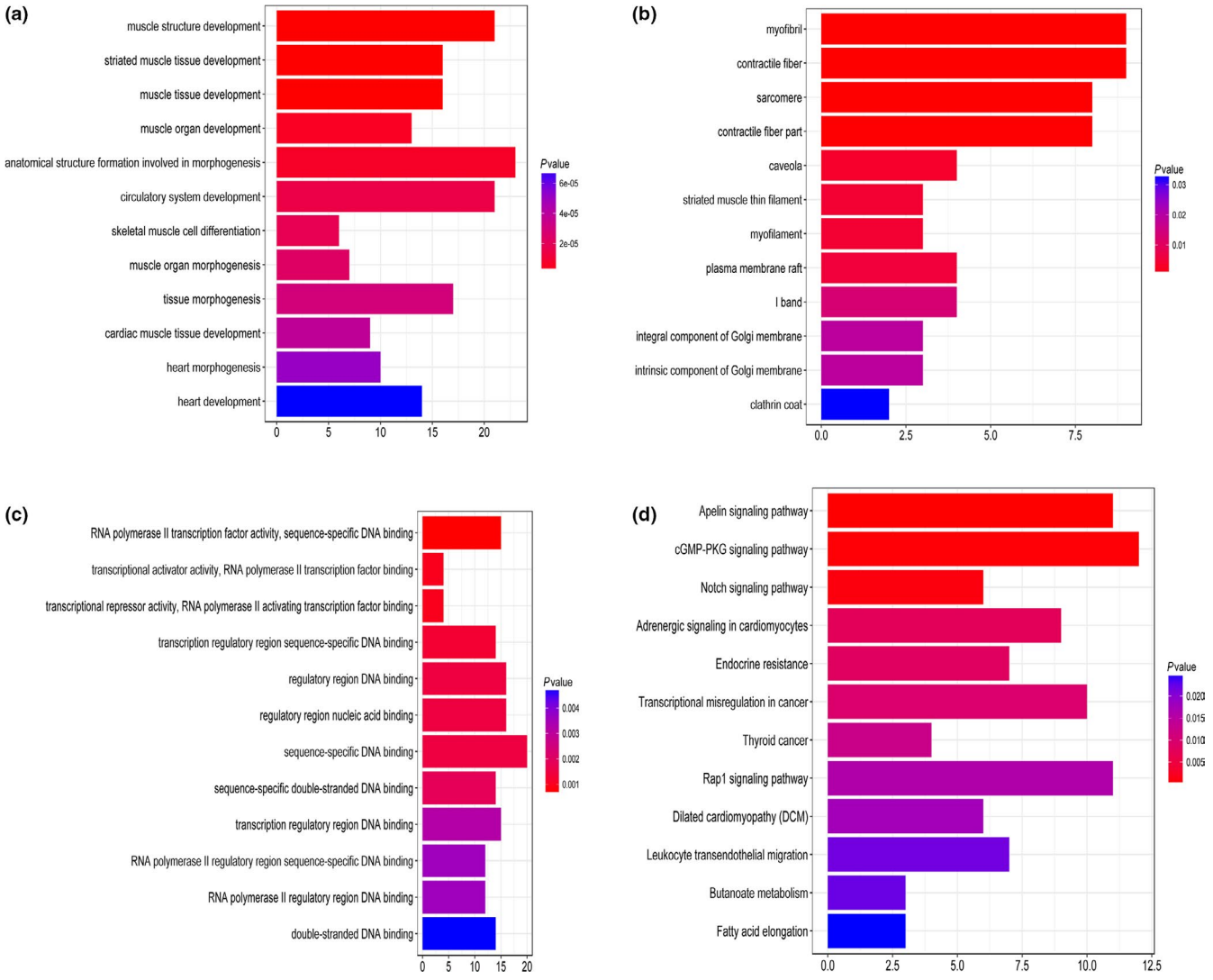

FIGURE 2 Function and pathway enrichment of differentially expressed genes. Top 12 pathways ordered by *p*-value. (a) Biological Process; (b) Cellular Component; (c) Molecular Function; (d) Kyoto encyclopedia of genes and genomes pathway. The ordinate represents the gene ontology function, and the abscissa represents the number of differential genes enriched to the term. *p*-values indicate the degree of enrichment, with smaller *p*-values indicating genes that are more likely to play significant functional roles

3.5 | The construction of PPI network

A total of 209 nodal genes and 409 pairs were explored in the protein interaction network map of DEGs (Figure 3). Table S2 lists the top 15 network nodes, which were the genes that scored the highest in topology properties.

A sub-network module was obtained when cytoscape MCODE was used with score > 6 (Figure 3). There were eight nodes (i.e. *TNNC1*, *MYL3*, *TCAP*, *TPM3*, *MYH7*, *TNNI1*, *MYH7B* and *TNNT1*) and 24 interaction pairs in the sub-network module, and four genes of the sub-network module (i.e. *TNNC1*, *MYL3*, *TCAP* and *TNNT1*) were associated with muscle quality.

3.6 | Variation in pig longissimus dorsi

Figure 4 shows the expression levels for using qRT-PCR DEGs obtained by RNA-Seq. Several genes, including *TNNC1*, *TNNT1* and *MYL3*, were significantly upregulated in pigs fed diets that were supplemented with mulberry leaves. However, no significant differences were found for the expression levels of *TCAP*, *ADCY2* or *HACD1*.

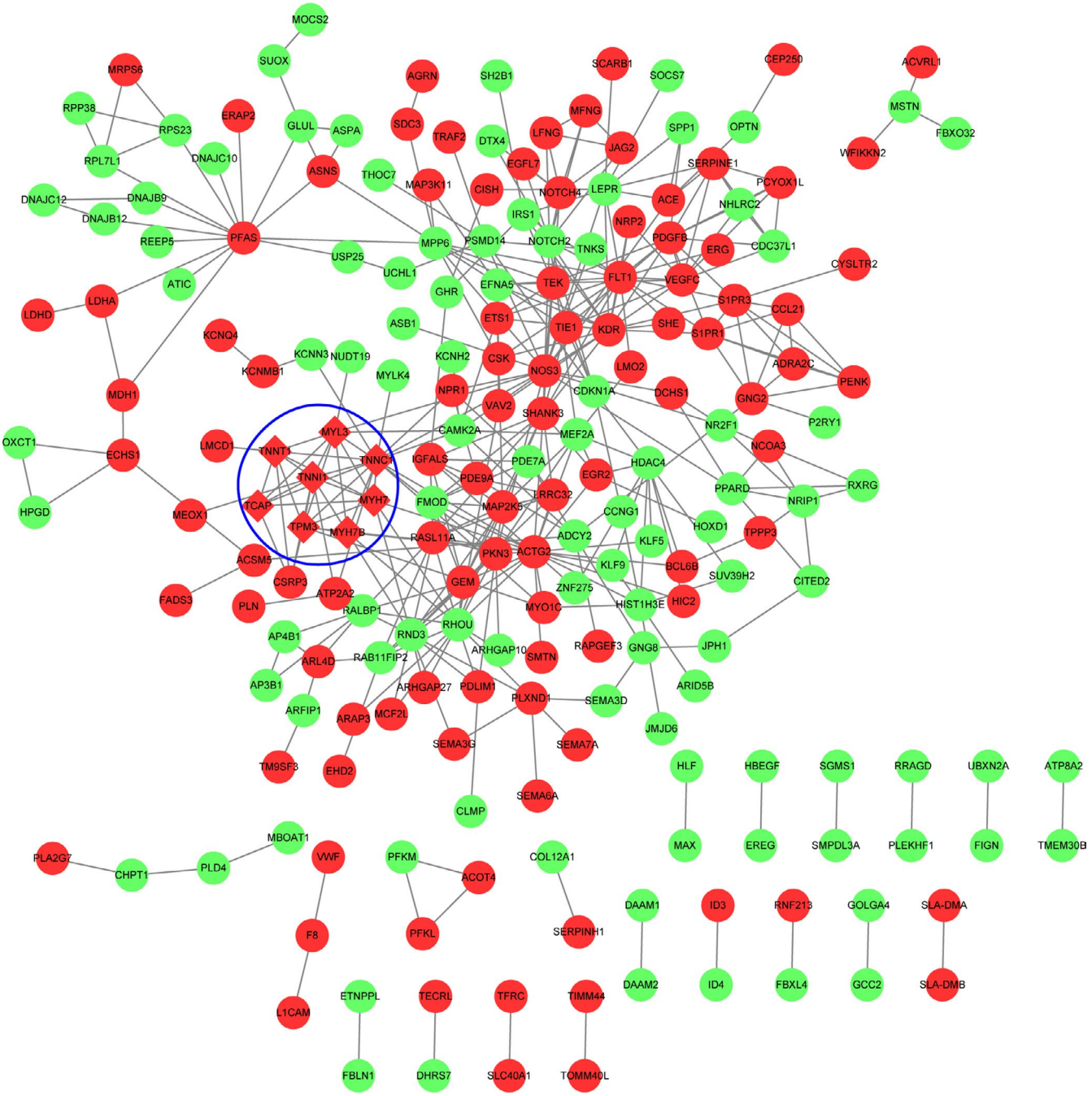

FIGURE 3 Protein–protein interaction network diagram. Network explored 209 nodal genes and 409 pairs. The red circular node represents upregulated genes, whereas the green circular node represents downregulated genes and the blue circular internal diamond node represents the sub-network module

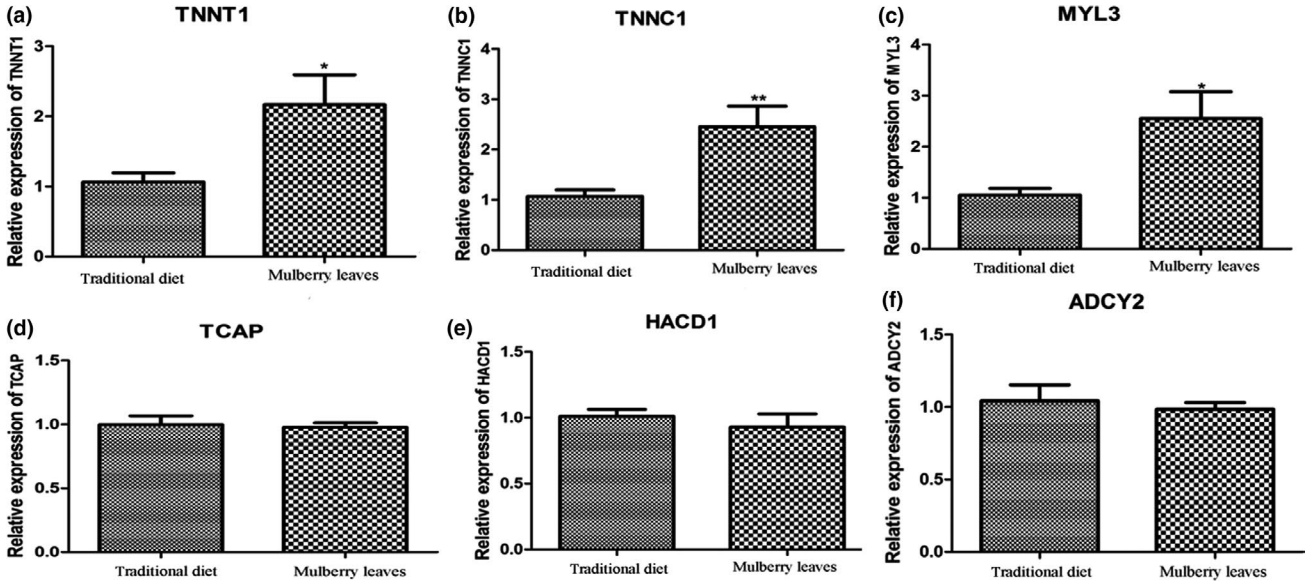

FIGURE 4 Validation of RNA-Seq data by quantitative real-time polymerase chain reaction. (a) TNNT1; (b) TNNC1; (c) MYCL3; (d) TCAP; (e) HACD1; (f) ADCY2. $^{**}p < 0.01$ versus traditional diet; $^{*}p < 0.05$ versus traditional diet

4 | DISCUSSION

The aim of the present study was to investigate the molecular mechanisms underlying the effect of dietary mulberry leaves on finishing pigs using high-throughput RNA-Seq technology. A total of 531 genes were differentially expressed in the mulberry-fed pigs, when compared to the control group, with 271 transcripts being upregulated and 260 being downregulated. Functional analysis demonstrated that DEGs were significantly enriched in functions related to muscle growth and development, and several genes (*ACOT4*, *ECHS1*, *HACD1*, *NPR1*, *ADCY2*, *MGLL* and *IRS1*) were enriched in the KEGG pathway associated with fatty acid metabolism. Furthermore, four of eight node genes, namely *TNNC1*, *MYL3*, *TCAP* and *TNNT1*, were associated with muscle formation and development, and the upregulation of *TNNC1*, *TNNT1* and *MYL3* was confirmed using qRT-PCR. Therefore, the inclusion of mulberry leaves in pig diets might be helpful for improving the muscle formation and development of pigs, and this likely occurs through the modulation of expression levels for three genes, namely *TNNC1*, *MYL3* and *TNNT1*.

With the development of high-throughput sequencing methods, the Illumina high-throughput sequencing platform was widely accepted for analysing both genomes and transcriptomes, due to its efficiency and accuracy. Meanwhile, when compared to traditional microarray hybridization, transcriptome analysis provides a more sensitive and accuracy platform, especially for interpreting the functional elements of genomes (Qiu et al., 2013; Wang et al., 2014). In the present study, a preliminary analysis of comparative transcriptome was designed to explore genes variation related with mulberry by RNA-Seq. This efficient deep-sequencing not only revealed pathways involved in muscle formation and development, but verified variation of gene expression in vivo.

A previous study that investigated the development of pig muscle tenderness found that the genes related to muscle tenderness were mainly involved in three functional networks, namely cell cycle, energy metabolism and muscle development (Lobjois et al., 2008). In the present study, GO analysis indicated that DEGs associated with dietary mulberry leaf supplementation were related to muscle and tissue structure. Furthermore, other previous studies have shown that polyphenols, such as those from hops and grapes, can improve growth and feed conversion, possibly by inhibiting inflammatory processes or affecting microbiota (Fiesel, Gessner, & Most, 2014; Gessner, Bonarius, & Most, 2017), and mulberry leaves probably contain polyphenols that could exert similar effects. Therefore, future studies could explore whether dietary polyphenol supplementation affects the expression of genes in pig muscle.

Muscle formation was tightly linked to meat quality, and genes related to muscle formation would be important in pig growth. Troponin T (TnT) is encoded by three homologous genes, which are specifically expressed in slow skeletal muscle (*TNNT1*), cardiac muscle (*TNNT2*) and fast skeletal muscle (*TNNT3*) (Amarasinghe, Hossain, & Jin, 2016; Novelli et al., 1992). Previous work has demonstrated that TnT, a 30- to 35-kDa myofilament protein, functions as an organizer in the calcium regulation process of muscle contraction and relaxation and that TnT interacts with TnI, TnC, tropomyosin and actin (Davoli et al., 2003). Recently, the gene was widely researched for its role in muscle function-related diseases, such as nemaline myopathy and Duchenne muscular dystrophy, as well as in resistance exercise in older adults (Baker et al., 2006; Zhang et al., 2010). Meanwhile, *TNNC1* was also one of the genes expressed in slow skeletal muscle. Such slow muscle-related genes are reportedly upregulated in mouse models that lack both dystrophin and the dystrophin homolog utrophin, when compared to mice that are only deficient in dystrophin (Baker et al., 2006). In contrast to one previous study, the present study found that *TNNT1* and *TNNC1*

were both significantly enriched in muscle contraction-related GO terms, and the two genes have also been shown as the validation of biomarkers for the quality of pork loin meat (Pierzchala et al., 2014). In the present study, both *TNNT1* and *TNNC1* were significantly upregulated, which could explain why the mulberry-fed pigs exhibited lower water loss rates and shear force values. Thus, the dynamic relationship between pork quality and the expression of *TNNC1* and *TNNT1* should be studied further, in order to confirm the role of the genes in pig associated with the effect of mulberry leaves.

In addition, the key role of *MYCL3* in muscle quality was indicated by PPI network analysis of the DEGs, and expression of the gene was also significantly elevated in pigs fed diets that were supplemented with mulberry leaves. GO analysis suggested that *MYL3* is mainly involved in cardiac muscle contraction. Ropka-Molik et al. (2014 used next-generation RNA-Seq technology to investigate two different pig breeds with different performance in terms of muscularity, growth rate and reproduction traits and found that *MYCL3* was one of most abundantly expressed genes in both breeds. Zhang et al. (2014 reported that the downregulation of *MYCL3* might indicate the negative regulation of intramuscular fat development, and the upregulation of *MYCL3* also appears to be associated with injury to skeletal muscle, possibly in order to facilitate muscle production (Burch et al., 2015). However, the general role of *MYCL3* in muscle production remains elusive. Although the significant higher expression levels of MYCL3 have been evaluated, the gene network associated with intramuscular fat developments should be further verified in animal trail.

The present study possesses several limitations that are worth noting. First, even though the study demonstrated the beneficial effects of dietary mulberry leaves, the number of pigs enrolled in the trial was small. Second, even though the results indicate the potentially significant modulatory role of *TNNC1*, *MYL3* and *TNNT1* in the development of meat quality in pigs, it is important that the molecular mechanism associated with the genes in pigs be verified in vivo.

5 | CONCLUSIONS

In conclusion, the data in our study presented here that mulberry leaves were benefit in pigs might mainly involve in muscle development, which might profit from the upregulated genes including *TNNC1*, *MYL3* and *TNNT1*. However, it should be noted that the dynamic relationship between muscle development of pig and the genes expression levels should be further studied to confirm the role of the genes in pig associated with the effect of mulberry leaves.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The US National Research Council's guidelines for the Care and Use of Laboratory Animals were followed.

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