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Effects of feeding conditions on gene expression in chicken breast muscle

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ABSTRACT. Chicken meat quality is becoming increasingly important among breeders and consumers. To understand the effect of feeding conditions on chicken meat quality, we investigated the profiles of genes expressed in chicken breast muscle. Using RNA sequencing, we identified 336, 321, and 387 differentially expressed genes among Chengkou, Daninghe, and Qingjiaoma chickens under scatter- and captivity-feeding conditions. Twenty-two genes differentially expressed between different feeding conditions were shown to be common among the three breeds. Seven of these genes were assessed by real-time quantitative PCR, which confirmed the findings of RNA sequencing and suggested that the results were viable. The differentially expressed genes showed enrichment for a series of significant pathways, including energy metabolism, xenobiotics biodegradation and metabolism, and the immune system. These results provide a solid foundation for elucidating the molecular mechanisms underlying chicken meat quality.

Key words: Chicken; Meat quality; Feeding conditions; Carcass; Genes expression

INTRODUCTION

In recent years, the consumption of chicken meat has increased and consumers have started to pay more attention to meat quality (Van Loo et al., 2014; Sans and Combris, 2015; Vorley and Lançon, 2016). The quality of chicken meat is affected by several factors, such as age, breed (Glamoclija et al., 2015), sex, and genotype of the bird (Djilali et al., 2016), as well as diet (Zhai et al., 2016), and feed conditions (HuiFeng et al., 2014). Meat quality can be assessed by a number of indices, including the concentration of inosine 5'-monophosphate (IMP) (Hamano and Kurimoto, 2016), intramuscular fat (IMF) content (Li et al., 2013a), pH, color, drip loss, cooking loss, and cooked meat shear force (Mudalal et al., 2015). Additionally, some customers prefer free-range meat because they believe that it has superior sensory qualities and improved taste (Latter-Dubois, 2001).

Many genes are associated with the quality of chicken meat (Liu et al., 2013), such as the fibroblast growth factor-binding protein (Felício et al., 2013) and fatty acid-binding protein (FABP) gene families (Li et al., 2006), *MYBPC1*, *CETP*, *GLTPD1*, *SNX4* (Cui et al., 2012), and *MSTN1* genes (Xu et al., 2015). Although many previous studies have investigated chicken meat quality, the mechanisms underlying these quality traits are unknown. We previously showed that IMP and IMF levels in chicken breast muscle differ significantly according to whether the chickens were fed under captivity-feed or scatter-feed conditions (HuiFeng et al., 2014). In the present study, we aimed to evaluate the effect of feeding conditions on gene expression in chicken muscle. To this end, we used high-throughput sequencing to identify the genes expressed in breast muscle from Chengkou (CK), Daninghe (DNH), and Qingjiaoma (QJM) chickens raised under captivity-feed and scatter-feed conditions. We then compared these gene expression profiles.

MATERIAL AND METHODS

Ethics statement

This study was approved by the Research Ethics Committee and the Animal Ethical Committee of Chongqing Academy of Animal Sciences. The experiments were performed in accordance with the Laboratory Animal Management Committee of Chongqing Academy of Animal Sciences and the Ministry of Science and Technology of the People's Republic of China (approval No.: 2006-398).

Animals

A total of 600-800 CK, DNH, and QJM female chickens were reared to the age of 5 months, fed the same diet, and provided water without the addition of antibiotics, growth promoters, hormones, or animal products in Wulong County, Chongqing, China (Table 1) (Gao et al., 2015). Two different feeding conditions were applied: scatter-feed and captivity-feed, which have been previously described (Gao et al., 2015). This gave a total of six groups: CK chickens under scatter-feed conditions (SFCK), CK chickens under captivity-feed conditions (CFCK), DNH chickens under scatter-feed conditions (SFDNH), DNH chickens under captivity-feed conditions (CFDNH), QJM chickens under scatter-feed conditions (SFQJM), and QJM chickens under captivity-feed conditions (CFQJM). We randomly selected five

female chickens from each group for use as the experimental animals. Breast muscle was collected from each chicken and immediately frozen in liquid nitrogen, then stored at -80°C for further study.

Table 1. Basic diet and nutrient levels.

Raw material	Content/%	Nutritional index	Nutritional level
Corn	65.00	Metabolizable energy	13.39
Soybean meal	23.00	Crude protein	18.00
Cottonseed meal	3.50	Lysine	0.85
Corn gluten meal	3.00	Egg + light	0.60
Grease	2.00	Ca/%	0.80
Limestone	1.00	P	0.65
$CaHPO_4$	1.20		
NaCl	0.30		
Premix	1.00		

Per kilogram premix ingredients: VA = 5000 IU/kg; VD = 31,000 IU/kg; VE = 10 IU/kg; VK = 0.5 IU/kg; pantothenic acid = 10 mg; niacin = 30 mg; VB1 = 1.8 mg; VB2 = 3.5 mg; VB6 = 3.5 mg; biotin = 0.1 mg; folic acid = 0.55 mg; choline = 1.0 g; VB12 = 0.012 mg; Mn = 80 mg; Zn = 60 mg; Fe = 80 mg; Cu = 8 mg; and Se = 0.15 mg.

RNA sequencing and genomic annotation

Total RNA was isolated from chicken breast muscle using Trizol reagent (15596-026; Invitrogen, Carlsbad, CA, USA). Isolated RNA was quantified by spectrophotometry and its integrity checked using 1% agarose gel electrophoresis to confirm that it was suitable for constructing cDNA libraries and for use in quantitative real-time PCR analysis. Total mRNA was purified using oligo (dT) magnetic beads, and cleaved into fragments (approximately 155 bp). Samples of total RNA from each group (5 x 2 mg) were pooled to construct cDNA libraries, which were sequenced using the Illumina HiSeq platform (Shanghai Personal Biotechnology Co., Ltd., Shanghai, China).

Clean reads were generated from raw data after removing adaptors, reads with uncertain base calls, low-quality bases, or reads shorter than 50 bp. Reads were then mapped and annotated against the chicken genome using the Bowtie2/TopHat2 software (Trapnell et al., 2009). We performed homology searches against Ensembl and searched for evolutionary genealogy using Non-supervised Orthologous Group (eggNOG) databases (Tatusov et al., 2003; Powell et al., 2012). We also predicted metabolic pathways and gene ontology of reads using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Gene Ontology (GO) websites, respectively. Reads per kilobase of exon model per million mapped reads were used to normalize read abundance (Mortazavi et al., 2008). RNA-seq databases generated from SFDNH, CFDNH, SFQJM, and CFQJM in this study were used to analyze genes that were affected by feeding conditions but not by breed, as shown in our previous study (Gao et al., 2015). In the present study, we integrated SFCK and CFCK chicken RNA-seq databases with the four previously published databases to analyze genes affected by feeding conditions (Gao et al., 2015).

Quantitative real-time PCR

The expression of seven genes that were differentially expressed in all three breeds (*HSP7*, *FOXO3*, *L-FABP*, *MUSTN1*, *CSRP3*, *ASNS*, and *MX*) was measured by quantitative real-time PCR (qRT-PCR). Optimal primers for the amplification of these genes were designed

using the NCBI website and synthesized by Invitrogen Biotechnology (Table 2). The chicken glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was selected as an internal control. qRT-PCR was performed using the ROX Reference Dye II kit (TaKaRa, Dalian, China) with the 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Table 2. Primer sequences used for qRT-PCR.

Symbol	Gene name	GenBank accession No.	Primer	Amplicon size (bp)
HSP70	Heat shock 70 kDa protein	AY178443.1	F: 5'- GCGAGTGGCTGACTGACCA-3'	230
			R: 5'-AAGTATGATGACCCCACA -3'	
FOXO3	Forkhead box O3	HGNC:3821	F: 5'-CGTTGTCAGTCTGAATGTGGGG-3'	122
			R: 5'-GACAGCAGATTTGGCAAAGGG-3'	
L-FABP	Fatty acid-binding protein, liver	NP_989523	F: 5'-GCAGAATGGGAATAAGTT-3'	212
			R: 5'-TTGTATGGGTGATGGTGT-3'	
MUSTN1	Musculoskeletal embryonic nuclear protein 1	Q76MS9	F: 5'-TGAAGGAGGAAGATCTCAAAGGA-3'	98
			R: 5'-GCCCATTTGTTCACACTGCTT-3'	
CSRP3	Cysteine and glycine-rich protein 3	NM_001199486	F: 5'-CTGTGGCAAATCGGTGTA-3'	130
			R: 5'-CTCTCCGTCTTTGTCTGTAAC-3'	
ASNS	Asparagine synthetase	NM_001030977	F: 5'-GCAGAAGAGAGCGAGAGG-3'	112
			R: 5'-GATGATCCAAAAACGGGA-3'	
MX	Interferon-induced GTP-binding protein Mx	AY695797	F: 5'-AAATGGCTCAAGAGGTGGA-3'	207
			R: 5'-CTTGCTGGATTGTGGAGGT-3'	
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_204305.1	F: 5'-GTGGTGCTAAGCGTGTCA-3'	182
			R: 5'-GGCTGGGATAATGTTCTGG-3'	

Statistical analyses

We used a fold-change of >2 and $P < 0.05$ as criteria to identify differentially expressed genes (DEGs) in pairwise comparisons among the groups. The expression of each gene relative to *GAPDH* was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

RESULTS

Gene expression analysis

The effects of feeding conditions on candidate genes for meat quality were assessed using the Illumina HiSeq 2000 platform (2 x 100 bp). In total, 13,349,711,000 raw data and 66,748,555 raw reads were obtained from six libraries (SFCK, SFDNH, SFQJM, CFCK, CFDNH, and CFQJM). After trimming and quality checking, 59,211,097 clean reads were recorded with 11,513,047,868 clean reads from the six groups (Table 3). There were 15,508 genes expressed in the six groups. More than 86% of the data were useful, and the raw data were filtered by Q20 or Q30 to more than 96 or 90%, respectively, which suggested that the results were reliable.

Differential gene expression analysis

We found that 336, 321, and 387 genes were differentially expressed between the SFCK and CFCK (**Table S1**), SFDNH and CFDNH (**Table S2**), and SFQJM and CFQJM groups, respectively (**Table S3**). Of these, 22 genes were common to all three pairwise comparisons, suggesting that their expression was affected by the feeding conditions (Figure 1 and **Table S4**). Seven of the 22 genes have been investigated in previous studies, and their structures are available on the NCBI website. However, the other genes were labeled as "uncharacterized

protein" or "transcript" (**Table S4**), and the intron/exon structures, and sequences of these genes were unknown, which made it difficult to design PCR primers. Therefore, we selected the abovementioned seven genes for qRT-PCR analysis to confirm the RNA sequencing findings (Figure 2). The results were consistent with those obtained by RNA sequencing, indicating that this technique is reliable.

Table 3. Sequencing and mapping of cDNA libraries from chicken breast muscle.

	SYCK	SYDNH	SYRZ	LYCK	LYDNH	LYRZ	Total
Useful data (%)	86.02%	86.17%	86.77%	86.02%	86.50%	85.92%	
Q20 (%)	96.54%	96.50%	96.67%	96.51%	96.60%	96.39%	
Q30 (%)	90.30%	90.13%	90.59%	90.21%	90.56%	90.17%	
Useful reads	21,124,372	19,649,360	20,905,912	18,386,148	19,468,504	18,887,898	
Mapped percent (%)	84.33%	85.82%	86.67%	85.99%	86.49%	87.02%	
Raw data (bp)	2,376,298,400	2,217,737,400	2,345,482,800	2,076,351,400	2,192,732,200	2,141,108,800	13,349,711,000
Raw reads	11,881,492	11,088,687	11,727,414	10,381,757	10,963,661	10,705,544	66,748,555
Clean data (bp)	2,044,167,938	1,911,115,133	2,035,140,809	1,786,150,261	1,896,738,173	1,839,735,554	11,513,047,868
Clean reads	10,562,186	9,824,680	10,452,956	9,193,074	9,734,252	9,443,949	59,211,097

SFCK = CK chickens under scatter-feed conditions; CFCK = CK chickens under captivity-feed conditions; SFDNH = DNH chickens under scatter-feed conditions; CFDNH = DNH chickens under captivity-feed conditions; SFQJM = QJM chickens under scatter-feed conditions; CFQJM = QJM chickens under captivity-feed conditions.

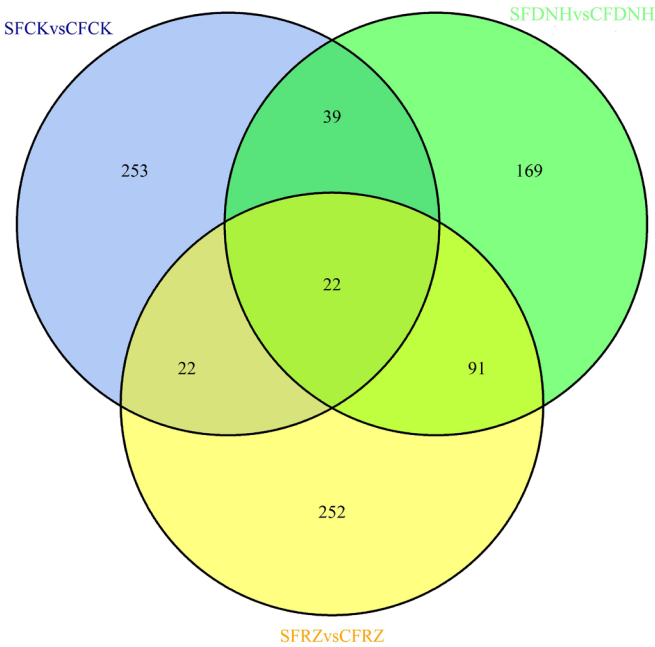

Figure 1. Summary of genes differentially expressed in chicken breast muscle. SFCK = Chengkou chickens raised under scatter-feed conditions; SFDNH = Daninghe chickens raised under scatter-feed conditions; SFQJM = Qingjiaoma chickens raised under scatter-feed conditions; CFCK = Chengkou chickens raised under captivity-feed conditions; CFDNH = DNH chickens raised under captivity-feed conditions; CFQJM = QJM chickens raised under captivity-feed conditions.

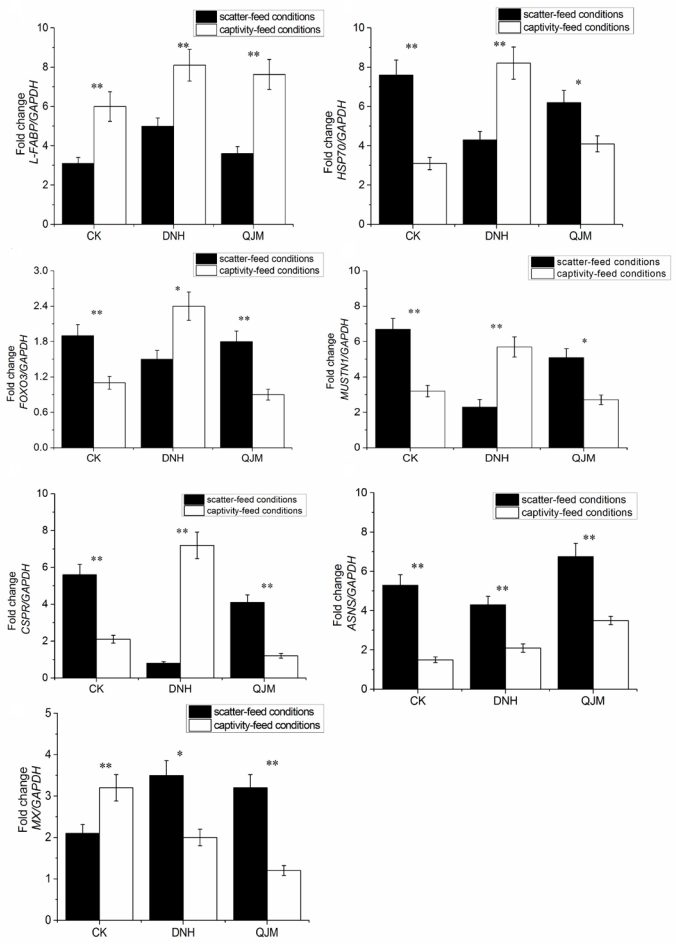

Figure 2. Real-time PCR validation of differentially expressed genes (DEGs) obtained from RNA-seq. **Highly significant at $P \leq 0.01$; *significantly different at $P \leq 0.05$.

FOXO3 is involved in many cellular activities, such as DNA damage repair, apoptosis, response to oxidative stress, cell growth and differentiation, cell cycle control, and energy metabolism (Medema et al., 2000; Kops et al., 2002; Tran et al., 2002; Sanchez et al., 2014). Previous studies have shown that *FOXO3* plays a crucial role in skeletal muscle development, and in the inhibition of proliferation and differentiation in duck myoblast skeletal muscle (Gan et al., 2016). This gene is also differentially expressed in chicken muscles with different glycogen contents, indicating that it could be involved in glycogen metabolism in chicken muscle (Sibut et al., 2011). The glycogen content in breast muscle was previously found to be related to growth rate and breast meat yield (Berri et al., 2001; Berri et al., 2007), as well as carcass fatness (Le Bihan-Duval et al., 2001; Sibut et al., 2008). Consistent with these findings, and with our earlier work in chickens (HuiFeng et al., 2014), we also showed that *FOXO3* mRNA expression differs according to the feed condition (Figure 2). We can infer from this that the feeding conditions affected the expression of *FOXO3*, leading to changes in glycogen metabolism in chicken muscle.

Musculoskeletal embryonic nuclear protein 1 (encoded by *MUSTN1*) regulates muscle development and growth in chickens (Li et al., 2012; Li et al., 2013b) and is involved in bone regeneration (Hadjiargyrou et al., 2002), myogenesis, cell differentiation, and muscle fiber fusion (Liu et al., 2010). The amino acid sequence of *MUSTN1* is evolutionarily conserved between birds and mammals, and *MUSTN1* may play an important role in breast muscle development in ducks (Xu et al., 2015). We observed the differential expression of *MUSTN1* under different feed conditions, suggesting that they affect *MUSTN1* expression and the process of muscle development.

GO categorization and KEGG terms analysis

For all three pairwise groups, the DEGs were classified into functional GO categories and KEGG terms. Functional analysis revealed that 86, 87, and 91 GO categories, and 31, 29, and 30 KEGG terms were mapped for the SFCK versus CFCK, SFRZ versus CFRZ, and SFDNH versus CFDNH comparisons, respectively. The top 10 significantly different GO categories and KEGG terms of the DEGs are shown in Figure 3.

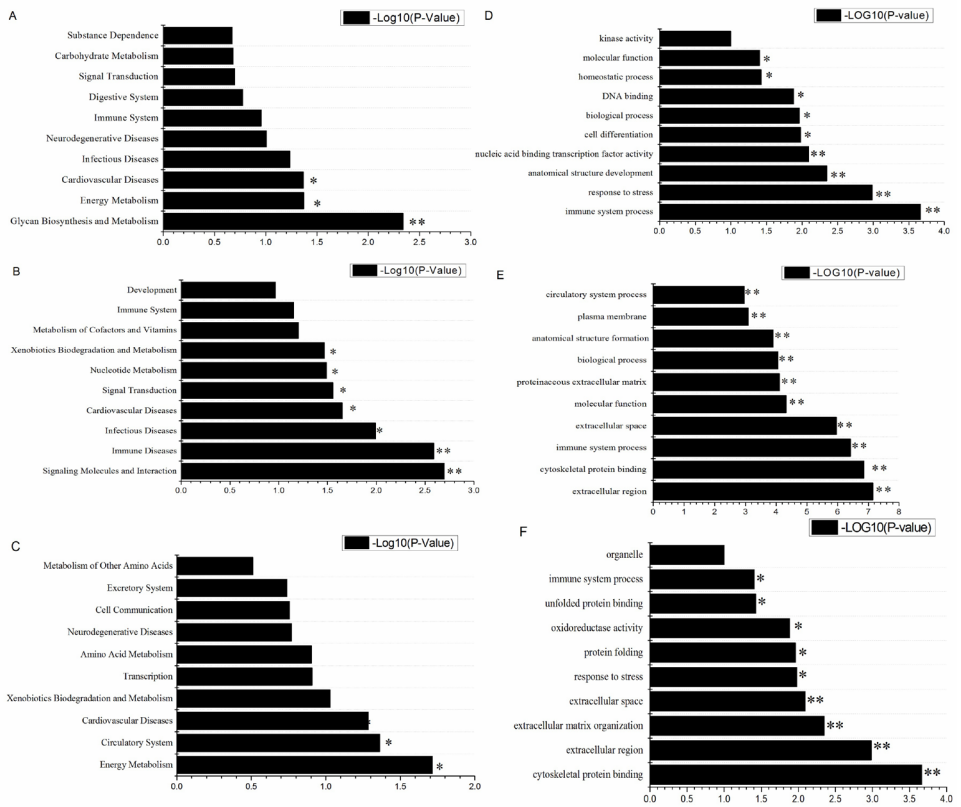

Figure 3. Top 10 KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) terms of the DEGs in chicken breast muscle. **A.** KEGG terms of SFRZ versus CFRZ; **B.** KEGG terms of SFCK versus CFCK; **C.** KEGG terms of SFDNH versus SFQJM; **D.** GO terms of SFRZ versus CFRZ; **E.** GO terms of SFCK versus CFCK; **F.** GO terms of SFDNH versus SFQJM. **Highly significant at $P \leq 0.01$; *significantly different at $P \leq 0.05$.

Energy metabolism, xenobiotic biodegradation and metabolism, and immune system pathways showed significant differences among all three pairwise comparisons, suggesting that they are affected by feed conditions. Similarly, the GO terms, response to stress, immune system process, and extracellular regions were significantly different in all three comparisons, also suggesting that they are affected by feed conditions.

DISCUSSION

In this study, next-generation sequencing technology was used to identify genes that are differentially expressed between CK, QJM, and DNH chickens reared under scatter-feed or captivity-feed conditions. Seven meat quality-related candidate genes (*L-FABP*, *HSP70*, *FOXO3*, *MUSTN1*, *CSRP3*, *ASNS*, and *MX*) were found to be differentially expressed in chicken breast muscle between the two feeding conditions, suggesting that they are important candidate genes of chicken meat quality. QJM is a broiler breed of chicken, while DNH and CK are local chicken breeds raised for both meat and eggs. The meat quality (IMP and IMF) of QJM chickens is significantly different from that of DNH and CK chickens (HuiFeng et al., 2014). In the present study, we found the trend of expression of some genes (*HSP70*, *FOXO3*, *MSUSTN1*, and *CBPR*) to be different in QJM chickens from in the other two groups (Figure 2). We suspect that these differences are associated with the special QJM breed of chicken.

mRNA levels do not always correlate with protein levels (Gygi et al., 1999) because complicated post-transcriptional regulation can occur (Blackinton and Keene, 2014). Therefore, RT-PCR analysis is limited in its ability to identify markers and mediators of cellular processes without additional tests.

According to our DEG annotations, several GO categorizations and KEGG terms differ significantly in response to feeding conditions, such as the immune system and response to stress. This study lays the foundation for elucidating the molecular mechanisms underlying chicken meat quality.

Conflicts of interest

The authors declare no conflicts of interest.

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Supplementary material

Table S1. Differently expressed genes between two feeding conditions in CK chickens.

Table S2. Differently expressed genes between two feeding conditions in Daninghe chickens.

Table S3. Differently expressed genes between two feeding conditions in Qingjiaoma chickens.

Table S4. Common differently expressed genes between two feeding conditions in CK, Daninghe and Qingjiaoma chickens.