Transcriptome analysis reveals normalization effect of nicotinamide and butyrate sodium on breast muscles of broilers under high stocking density

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Abstract

Background In recent years, increased attention has been focused on breast muscle yield and meat quality in poultry production. Supplementation with nicotinamide and butyrate sodium can improve the meat quality of broilers. However, the potential molecular mechanism is not clear yet. This study was designed to investigate the effects of supplementation with a combination of nicotinamide and butyrate sodium on breast muscle transcriptome of broilers under high stocking density.

Methods A total of 300 21-d-old Cobb broilers were randomly allocated into 3 groups based on stocking density: low stocking density control group (L; 14 birds/m²), high stocking density control group (H; 18 birds/m²), and high stocking density group provided with a combination of 50 mg/kg nicotinamide and 500 mg/kg butyrate sodium (COMB; 18 birds/m²), raised to 42 days of age.

Results The H group significantly increased cooking losses, pH decline and activity of lactate dehydrogenase in breast muscle while COMB showed a significant decrease in these indices (P < 0.05). The transcriptome results showed that key genes involved in glycolysis, proteolysis and immune stress were up-regulated whereas those relating to muscle development, cell adhesion, cell matrix and collagen were down-regulated in the H group. In contrast, genes related to muscle development, hyaluronic acid, mitochondrial function, and redox pathways were up-regulated while those associated with inflammatory response, acid metabolism, lipid metabolism, and glycolysis pathway were down-regulated in the COMB group.

Conclusions The combination of nicotinamide and butyrate sodium may improve muscle quality by enhancing mitochondrial function and antioxidant capacity, inhibiting inflammatory response and glycolysis, and promoting muscle development and hyaluronic acid synthesis.

Background

Intensive stocking in the rapidly developing poultry industry worldwide has become a norm. However, high stocking density causes oxidative stress in broilers [1] and reduces the tenderness and increases the drip loss of breast muscle [3, 4]. Oxidation is one of the leading reasons for the deterioration of meat quality [2], and oxidative stress causes protein and lipid peroxidation as well as cellular damage [5, 6] which ultimately affects meat quality [7]. Nicotinamide (NAM) reduces oxidative stress and inhibits reactive oxygen species (ROS) production [8, 9]. Dietary supplementation with NAM has been observed to minimize the formation of carbonylated proteins in the liver of high-fat fed mice [10]. Butyrate sodium (BA) could also improve antioxidant capacity in a human study [11]. Further, the addition of BA can enhance the activities of superoxide dismutase and catalase and reduce the level of malondialdehyde in serum [12]. Butyrate treatment has been reported to decrease the levels of markers of oxidative stress and apoptosis in mice [13]. As treatment with NAM and BA both can elevate antioxidant capacity and muscle function, it may improve the muscle quality of broilers under high stocking density. Dietary supplementation with 60 mg/kg niacin (NAM precursor) reduces the drip loss of breast muscles in broilers [14]. Dietary supplementation with BA can increase broiler weight, decrease abdominal fat percentage [15], and reduce intramuscular fat content [16].

Mitochondrial biogenesis has previously been associated with preservation of muscle mass and beneficial effects on metabolism [17]. Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) is a crucial regulator of mitochondrial biogenesis. Replenishment with nicotinamide adenine dinucleotide (NAD) induces mitochondrial biogenesis by increasing PGC1α expression [18, 19]. NAM is the primary source of NAD which is obtained through the salvage pathway. As a precursor of NAD, treatment with NAM also enhances PGC-1α expression [20]. Impaired intramuscular NAD synthesis compromises skeletal muscle mass and strength over time, which can be quickly restored with an oral NAD precursor [21]. Besides, NAD biosynthesis alleviates muscular dystrophy in a zebrafish model [22] and promotes muscle function in Caenorhabditis elegans [23]. Addition of niacin (precursor of NAM) has been reported to increase the number of oxidative type I fibres in skeletal muscles of growing pigs [24] and induce type II to type I muscle fibre transition in sheep [25]. Further, supplementation with butyrate increases mitochondrial function and biogenesis of skeletal muscle in mice and rats [26, 27]. Further, the intake of BA increases the percentage of type 1 fibres [26, 28] and muscle fibre cross-sectional area in skeletal muscle [13].

Although supplementation with NAM or BA alone can elevate antioxidant capacity and improve the meat quality of broilers, the effect of combined supplementation with NAM and BA on the meat quality of broilers is not clear yet. Therefore, we performed transcriptome sequencing of broiler breast muscles to elucidate the molecular mechanism of the effect of feeding density and nutrient regulation on meat quality.

Results

Meat quality

Compared with the L group, the H group showed significantly increased cooking loss of breast muscle (P < 0.05). The COMB group showed decreased cooking loss compared with the H group (P < 0.05). Besides, the drip loss in the COMB group was lower than that in the L group, as
well (P < 0.05) (Figure 1).

The 45-min pH value in the H group was higher than that in the other 2 groups (P < 0.05) while there was no significant difference in 24-h pH values among the groups. Thus, the pH decline during 45 min to 24 h in the H group was significantly higher than that in the other 2 groups, indicating that the H group had rapid pH drop rate, which was attenuated in the COMB group under high stocking density (Figure 2).

**Anti-oxidant capacity**

The activity of LDH in the H group was higher (P < 0.05) than that in the L group. The COMB group had significantly decreased (P < 0.05) activity of LDH when compared with the H group. However, stocking density had no significant effect on the activities of CK, T-AOC, MDH, anti-superoxide anion and the content of hydroxyproline (Table 3).

**RNA sequencing data and differentially expressed genes (DEGs)**

In the principal component analysis (PCA), there was a clear divergence among the H, L and COMB groups. In the Venn diagram, the number of identified genes in the H, L and COMB were 11777, 12554 and 11633, respectively (Figure 3). Compared with the H group, the number of DEGs in the L group and COMB group were 3752 and 773, respectively (Figure 4).

The gene sets were produced by DEGS. From Venn analysis of genes sets, we found that there were 1310 genes shared in common between the COMB group and the L group. Nevertheless, there were only 6 genes owed by both the COMB group and the H group. Similarly, from the iPath map of metabolic pathways, there were a total of 830 pathways annotated in common. In contrast, there was only 1 pathway owed by both the COMB group and the H group (Figure 5).

**Up-regulated genes in the H group**

Compared with those in the L group, a total of 1894 genes were up-regulated in the H group (Figure 4), which were mainly involved in muscle contraction, cell localization, ion transport, lipid metabolism, glycolysis, proteolysis, and immune stress (Figure 6).

Muscle contraction-related pathways were enriched in the H group. They involved vital genes including MYLK2, NOS1, TMOD4, and Six1 (Table 4). The H group was enriched for cell-localization-related genes such as KEAP1, CDKN1A, ERBB4, and TMD4 (Table 4). Additionally, high-density up-regulated ion and amino acid transport-related genes included KCNJ12, KCNA7, SLC38A3 and SLC38A4, which are involved in ion transmembrane transport and transporter activity (Table 5). High-density enriched glycolysis-related pathways included fructose metabolism, fructose-2,6-diphosphate 2-phosphatase activity, and fructose 2,6-diphosphate metabolism (Table 6). The lipid metabolism-related genes such as MID1IP1, ACACB and Lpin1 were up-regulated in H group, which are involved in lipid synthesis and lipid oxidation (Table 6).

Stress response pathways including non-biologically stimulated cellular responses, extracellular stimuli response and nutritional level response were also enriched in the H group. Furthermore, high-density up-regulated proteolysis-related genes include TINAG, USP24, OTUD1, KEAP1, KLHL34, and SMCR8. Also, high-density enriched immune pathways include the regulation of host defence responses to viruses and prostaglandin receptor-like binding (Table 7).

In Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, genes involved in calcium signalling pathway (RYR), inflammatory mediator regulation of RTP channels (PLA2) and chemokine signalling pathway (SOS) (Fig. S1-S3) were enriched in the H group.

**Down-regulated genes in the H group**

Compared with those in the L group, a total of 1858 genes were down-regulated in the H group (Figure 4), which were involved in cell adhesion, cell matrix, and cell migration, etc (Figure 7).

The genes involved in muscle development include muscle fibre assembly and binding (LMOD2, MYOZ2 and ACTN1, etc.) and muscle fibre development (DSG2, LMOD2 and FSCN1, etc.), which were down-regulated in H group (Table 8). High-density also down-regulated genes related to cell-matrix pathways such as MMP9, FBLN1, THBS4, and VCAN. High-density also down-regulated collagen synthesis and collagen binding related genes including ADAMTS3, ADAMTS14, COL1A2, and LUM (Table 9). Besides, the adhesion-associated genes including DSG2, CSTA, THY1, TGFβ1, NOV, CDH11 and FN1 were diminished. Additionally, antioxidant genes including MGST2, PTGS2, NCF1, SOD3, and CYBB were also down-regulated (Table 10).

In KEGG enrichment analysis, down-regulated genes in the H group were involved in ECM-receptor interaction (COL1A3, THBS1, FN1, TN, ITGA5, ITGA8 and ITGB8), adherens junction (SHP-1, TGFβR, α-Actinin and Slug) and focal adhesion (Actinin and MLC) (Fig. S4-S6).

**Up-regulated genes in the COMB group**
Compared with those in the H group, up-regulated genes in the COMB group were involved in muscle development, hyaluronic acid synthesis, mitochondrial function, and redox pathway (Figure 8).

The muscle development-related pathways enriched in the COMB group included positive regulation of muscle tissue development and muscle cell decision processes, which involved key genes such as MYF6, LMCD1 and TRPC3. Besides, the COMB group was enriched for mitochondria-associated pathways such as electron transport chains, mitochondrial respiratory chain complex I and mitochondrial protein complex pathways, which involved genes including TOMM6, NDUFV1, NDUFS5, NDUFB2, NDUF2, LMCD1, ZNF593 and COASY (Table 11). The hyaluronic acid-related genes up-regulated in the COMB group included HYAL1 and HYAL3. Besides, the redox-related genes including LDHD, CPOX, SUOX, NDUFV1, GRHPR, DOHH and NDUF2 were up-regulated in the COMB group, which were involved in the pathways such as redox process, NAD binding, NADPH binding and NADH dehydrogenase complex (Table 12). In KEGG enrichment analysis, up-regulated genes in the COMB group were involved in oxidative phosphorylation (NDUFS5, NDUVF1, NDUF2, NDUF13, NDUF12, NDUF7 and NDUC2) (Fig. S7).

**Down-regulated genes in the COMB group**

Compared with those in the H group, down-regulated genes in the COMB group were involved in the inflammatory response, acid metabolism, fatty acid metabolism, and glycolysis-related pathways (Figure 9).

The inflammatory response-related genes down-regulated in the COMB group included CCR5 and ALOX5 while the immune response-related genes included C1S, BLK, CCR5 and MARCH1 (Table 13). The acid metabolism-related pathways include organic acid synthesis process, oxoacid metabolism process and carboxylic acid synthesis process, which involved genes such as PSAT1, SCD, MAT1A, ALOX5, ST3GAL1 and ALDOB. The genes involved in fatty acid metabolism pathways include SCD and ALOX5. In addition, down-regulated genes in the COMB group were involved in glycolytic and carbohydrate metabolism, which included GALNT16, ST3GAL1, ALDOB and MAT1A (Table 13).

In KEGG enrichment analysis, genes involved in the regulation of lipolysis in adipocytes (PLIN), glycolysis/gluconeogenesis (ALDO) and arachidonic acid metabolism (ALOX5) were down-regulated in the COMB group (Fig. S8-S10).

**Transcriptome differential gene verification**

The transcriptome differential genes were verified by real-time PCR, and the gene expression pattern was consistent with the transcriptome results (Figure 10).

**Discussion**

In the current study, the H group showed significantly increased cooking loss of breast muscle when compared with the L group. The muscle disease such as PSE (Pale, Soft and Exudative) meat [36] and wooden breast [37] have higher cooking loss than normal meat.

Stress is an essential cause of the decline in meat quality. In this study, the activity of LDH in the H group was higher than that in the L group. In transcriptome analysis, the enriched genes in the H group were involved in stimuli response pathway. In the H group, genes encoding nitric oxide synthase 1 (NOS1), Kelch-Like ECH-associated protein 1 (KEAP1) and cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A) were up-regulated. High levels of NO reduce the antioxidant capacity of post-mortem muscles, increasing the accumulation of ROS and reactive nitrogen, resulting in high levels of protein oxidation. Studies have shown that inhibition of nitric oxide synthase can significantly reduce protein carbonyl content and protein oxidation [38]. Inhibition of CDKN1A expression by miRNAs promotes myoblast proliferation [39]. Up-regulation of KEAP1 expression increases the degradation of Nrf2 in cells, making cells more susceptible to free radical damage [40]. Heat stress can reduce the oxidative stability of broiler muscle protein and reduce the strength of the myofibrillar gel, resulting in increased drip loss and cooking loss in broilers [41]. A study has shown that genes involved in the stimulation response pathway are significantly enriched in muscles with high drip loss [42]. Therefore, increased expression of stress pathway-related genes such as KEAP1 and CDKN1A may be one of the causes of muscle quality deterioration.

This study found that the H group had the fastest pH decline rate. The rapid decline in pH is usually accompanied by an increase in the rate of glycolysis and the accumulation of lactic acid, resulting in a decrease of muscle function [43]. In this study, high stocking density led to up-regulation of genes involved in glycolysis and fat metabolism pathways. Anaerobic glycolysis is a vital energy metabolism pathway for post-mortem broilers. Under anaerobic conditions, muscle glycogen degradation occurs through glycolysis, which causes pyruvate to synthesize lactic acid, thus leading to a decrease in muscle pH due to the accumulation of lactic acid [44, 45]. High stocking density in this study also caused up-regulation of striated muscle contraction pathway-related genes such as SIX homeobox 1 (Six1). It has been found that white streak muscles have up-regulated expression of striated muscle contraction-related genes compared with normal meat [46]. Six1 converts slow muscle fibres into fast muscle fibres [47, 48]. The proportion of fast muscle fibres was negatively correlated with post-mortem pH [49]. Besides,
the enriched genes in the H group were involved in calcium transport, sodium transport, and cation transport. Importantly, ion balance is the basis for maintaining normal physiological functions. Abnormal metabolism caused by high concentrations of calcium ions may be associated with the incidence of turkey PSE [50]. Furthermore, changes in muscle cation homeostasis may mark the beginning of muscle degeneration [51] and cause a reduction in meat quality [52].

Dietary supplementation with niacin (nicotinamide precursor) at 60 mg/kg was reported to reduce the drip loss of breast muscles in broilers [14]. In our study, the COMB group showed significantly reduced drip loss and cooking loss compared with the H group. Further, the COMB group showed significantly decreased activity of LDH compared to the H group. Besides, the COMB group showed inhibited expression of glycolytic and inflammation genes [43].

In KEGG enrichment analysis, the enriched genes in the H group were involved in inflammatory mediator regulation of RTP channels and chemokine signalling pathway. In contrast, the up-regulated genes in the COMB group were involved in the inflammatory response. Macrophage infiltration in the pectoral muscle might cause muscle damage [53]. The muscle disease such as white striped muscle is usually accompanied by elevated expression of immune-related genes [46]. During tissue degeneration, immune cells immediately enter the site of injury, triggering an inflammatory response, and attracting more immune cells to the damaged area. It can cause phagocytosis of cell debris and release of cytokines, prostaglandins and other signalling proteins, resulting in interstitial spaces [54].

We found that key genes down-regulated in the H group, such as MYOZ2, were involved in muscle development, cell adhesion, cell matrix, collagen, and cytoskeleton. MYOZ2 belongs to sarcomeric family and links calcineurin to alpha-actinin at the Z-line of skeletal muscle sarcomere and can play a role in skeletal muscle differentiation and growth [55]. It was suggested that MYOZ2 knockout mice had neuromuscular disease [56]. Also, genes down-regulated in the H group were involved in cell matrix and collagen pathways. Extracellular matrix (ECM) is a major macromolecule in skeletal muscle and has a substantial effect on meat quality. The remodelling of ECM is mainly regulated by matrix metalloproteinases. The expression of matrix metalloproteinase-1 is negatively correlated with cooking loss and positively correlated with hydraulic performance [57]. Collagen is an abundant connective tissue protein that is an important factor in the tenderness and texture of the meat and is well resistant to physical damage during cooking [58]. The addition of collagen increases the ability of pork [59] and poultry [60] to combine with water and reduces cooking losses. Furthermore, high stocking density downregulates cell adhesion, cytoskeletal and integrin binding-related genes such as integrin subunit alpha 8 (ITGA8), integrin subunit beta 8 (ITGB8) and integrin subunit beta like 1 (ITGBL1). Proteolytic degradation of cell adhesion proteins is associated with the production of drip channels [61]. The cytoskeleton is a highly complex network composed of a large number of connections between myofibrils and myofibrillar membranes. Degradation of the cytoskeleton causes extracellular water to flow into the muscle cells, thereby increasing drip loss [62]. Integrins are heterodimeric cell adhesion molecules that bind the extracellular matrix to the cytoskeleton and play an essential role in controlling cell membrane-cytoskeletal attachment and signalling pathways [63]. The β1 integrin is responsible for the attachment of the cell membrane to the cytoskeleton [64]. Degradation of β1 integrin promotes the formation of water channels between cells and cell membranes, thereby increasing drip loss [65]. In addition, it has been found that integrins are inversely related to pork drip loss [66].

Compared with the H group, the COMB group showed up-regulation of muscle development, hyaluronic acid levels, mitochondrial function, and the redox pathway. Studies have found that hyaluronic acid is a crucial water-holding molecule [67, 68]. Furthermore, supplementation with antioxidant isoflavones can be achieved by reducing lipid peroxidation and increasing oxidative stability in the pectoral muscles [69]. Therefore, enhanced hyaluronic acid biosynthesis and antioxidant capacity may improve muscle quality.

Additionally, up-regulated genes in the COMB group involved the complex I-related gene NDUFS5. The mitochondrial respiratory chain (MRC) consists of four membrane-bound electron transport protein complexes (I-IV) and ATP synthase (complex V) that produce ATP for cellular processes. Complex I deficiency, NADH ubiquinone oxidoreductase is the most common form of MRC dysfunction and is associated with a variety of diseases [70, 71]. Complex I deficiency leads to various physiological disorders such as ATP depletion, calcium homeostasis, ROS accumulation [72] and induction of apoptosis [73]. A study found that mitochondrial and oxidative phosphorylation-related gene expression was negatively correlated with drip loss. A negative correlation with drip loss means that there is a decrease in the number of mitochondria in muscles with high drip loss [74].

**Conclusion**

High stocking density may cause oxidative stress, abnormal muscle contraction, and abnormal metabolism of glycolipids; destroy ion channels and cell matrix; reduce muscle strength by inhibiting muscle development, and cell adhesion and collagen synthesis, all of which result in reduced muscle function. Supplementation with NAM and BA in combination can improve mitochondrial function and antioxidant capacity, and inhibit inflammatory response and glycolysis by promoting muscle development and hyaluronic acid synthesis, thereby reducing drip loss of the breast muscle and improving muscle quality (Figure 11).
Methods

Experimental birds, diets, and management

A total of 300, 21-day-old Cobb broilers, were randomly divided into 3 groups: low stocking density (L, 14 birds/m²), high stocking density (H, 18 birds/m²) and combination of NAM and BA (COMB, 18 birds/m²), with 6 replicates for each group. The L and H groups were fed a basal diet. The COMB group was fed basal diet supplemented with 50 mg/kg NAM and 500 mg/kg BA.

NAM (99% purity, Jiangxi Brothers Medicine Co. Ltd., China) and BA (encapsulated, 30% effective content, Hangzhou King Technology Feed Co. Ltd., China) were purchased from the market. The composition and nutrient levels of basal diet are shown in Table 1. Experimental diets were formulated to meet or exceed the minimum nutrient requirements recommended by the National Research Council (1994) [29].

This study was conducted in an experimental chicken farm of the College of Animal Science and Technology, China Agricultural University. Broilers were raised from 21 to 42 days of age, and feed and water were provided ad libitum. The temperature was maintained at 20-21 °C throughout the experiment while the illumination period was 18 h per day.

Sample collection

At 42-day, after 5 h of starvation, 1 broiler per replicate was randomly selected and euthanized by intravenous injection of pentobarbital sodium (390 mg/ml) at a dose of 300 mg/kg. The breast muscle of each broiler was collected and put into liquid nitrogen immediately, then stored at -80°C until further analysis. Each group had six replicates for the determination of meat quality, enzyme activities and mRNA relative expression; there were three biology replicates in each group for RNA-sequencing.

Meat quality analysis

After slaughtering, the right side of the major pectoral muscle was quickly removed for meat quality evaluation, including drip loss, cooking loss and pH. For the determination of drip loss, approximately 10 g muscle was weighed (W1) and placed in a sealed polyethylene bag at 4°C. The muscle was reweighed (W2) after 24 h, and drip loss was expressed as (W1–W2) / W2 * 100% [30]. Cooking loss was determined according to the method described by Cai et al. [31]. Cooking loss of samples was calculated as: (initial weight-final weight)/initial weight × 100%. The pH values of the pectoral muscle at 45 minutes and 24 hours were measured by a pH meter (testo 205; Germany). Each sample was tested at 3 different locations (top, middle and bottom) and the average of 3 measurements was calculated.

Enzyme Activity Determination in breast muscle

The activities of total antioxidant capacity (T-AOC, cat#A015), lactate dehydrogenase (LDH, cat#A020-2), creatine kinase (CK, cat#A032), malic dehydrogenase (MDH, cat#A021-2), anti-superoxide anion (cat#A052) and the content of hydroxyproline (cat# A030-2) in breast muscle were measured with commercial analytical kits according to the manufacturer's recommendations (Jian Cheng Bioengineering Institute, Nanjing, China).

RNA extraction

Total RNA was extracted from the breast muscle using TRIzol® Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Then RNA quality was determined by 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified using the ND-2000 (Nanodrop Technologies, Wilmington, Delaware). Only high-quality RNA sample (OD260/280=1.8~2.2, OD260/230≥2.0, RIN≥6.5, 28S:18S≥1.0, >10μg) was used to construct a sequencing library.

Library preparation and Illumina Hiseq xten Sequencing

RNA-seq transcriptome library was prepared following TruSeqTM RNA sample preparation Kit from Illumina (San Diego, CA, USA) using 5μg of total RNA. Shortly, messenger RNA was isolated according to the polyA selection method by oligo (dT) beads and then fragmented by fragmentation buffer firstly. Secondy, double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA, USA) with random hexamer primers (Illumina). Then the synthesized cDNA was subjected to end-repair, phosphorylation and ‘A’ base addition according to Illumina's library construction protocol. Libraries were size selected for cDNA target fragments of 200-300 bp on 2% Low Range Ultra Agarose followed by PCR amplified using Phusion DNA polymerase (NEB) for 15 PCR cycles. After quantified by TBS380, paired-end RNA-seq sequencing library was sequenced with the Illumina HiSeq xten (2 × 150bp read length).

Read mapping
The raw paired-end reads were trimmed and quality controlled by SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) with default parameters. Then clean reads were separately aligned to the reference genome with orientation mode using TopHat version2.0.0 (http://tophat.cbcb.umd.edu/) [32] software. The mapping criteria of bowtie was as follows: sequencing reads should be uniquely matched to the genome allowing up to 2 mismatches, without insertions or deletions. Then the region of the gene was expanded following depths of sites and the operon was obtained. Also, the whole genome was split into multiple 15kbp windows that share 5kbp. New transcribed regions were defined as more than 2 consecutive windows without the overlapped region of genes, where at least 2 reads mapped per window in the same orientation.

**Differential expression analysis and Functional enrichment**

To identify DEGs (differentially expressed genes) between two different samples, the expression level of each transcript was calculated according to the fragments per kilobase of exon per million mapped reads (FRKM) method. RSEM (http://deweylab.biostat.wisc.edu/rsem/) [33] was used to quantify gene abundances. R statistical package software EdgeR (Empirical analysis of Digital Gene Expression in R, http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) [34] was utilized for differential expression analysis. Besides, functional-enrichment analysis including GO and KEGG were performed to identify which DEGs were significantly enriched in GO terms and metabolic pathways at $P$-value $\leq 0.05$ compared with the whole-transcriptome background. GO functional enrichment and KEGG pathway analysis were carried out by Goatools (https://github.com/tanghaibao/Goatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do) [34].

**Muscle developmental gene**

The cDNA was synthesized by using a reverse transcription kit PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (RR047A, TAKARA, Japan). Then it was stored in a -80 °C refrigerator. Fluorescence quantitative PCR was performed according to the instructions of TB Green™ Premix Ex Taq™ (Tli RNaseH Plus) (RR420A, TAKARA, Japan). The reaction apparatus was a 7500 fluorescence detection system (Applied Biosystems), and the PCR reaction conditions were as follows: after pre-denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s and 60 °C for 34 s. After the completion of PCR amplification, the dissolution curve was observed, and agarose gel electrophoresis was performed to identify whether the amplified gene fragment conformed to the design length, and the specificity of the amplification product was verified. The target gene and the internal reference gene beta-actin primer sequence are shown in Table 2. The results of gene expression were analyzed and compared using $2^{\Delta \Delta CT}$.

**Statistical analysis**

The results are expressed as means with their standard error mean (SEM). One-way ANOVA was used for single factor analysis by SPSS 20.0 for Windows (SPSS Inc. Chicago, IL). Differences were considered significant at $P < 0.05$.

**Abbreviations**

L: low stocking density group; H: high stocking density group; COMB: a combination of nicotinamide and sodium butyrate group; NAM: nicotinamide; ROS: reactive oxygen species; BA: butyrate sodium; PGC1α: peroxisome proliferator-activated receptor-γ coactivator 1α; NAD: nicotinamide adenine dinucleotide; T-AOC: total antioxidant capacity; LDH: lactate dehydrogenase; CK: creatine kinase; MDH: malic dehydrogenase; DEGs: differentially expressed genes; NOS1: nitric oxide synthase 1; KEAP1: Kelch-Like ECH-associated protein 1; CDKN1A: cyclin-dependent kinase inhibitor 1A.

**Declarations**

**Conflict of Interest**

All authors declare no conflicts of interest.

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**Availability of data and materials**
All the sequencing data are deposited in SRA under the Bioproject accession number PRJNA558637.

Authors’ contributions

JMY conceived and designed the experiment; YQW, YLW, DFY performed the experiments. YQW analyzed RNA Sequencing data and drafted the manuscript. TM and JMY reviewed and edited the manuscript. All authors critically revised the manuscript for important intellectual content and all approved the final version of this manuscript.

Ethics approval and consent to participate

All procedures used in our experiments were approved by the Institutional Animal Care and Use Committee of the China Agricultural University (Beijing, China, permit number SYXK20130013).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table 1. The composition and nutrient level of basal diet

| Ingredient                  | %   | Nutrients                     | %     |
|-----------------------------|-----|-------------------------------|-------|
| Corn                        | 62.05 | Metabolic energy             | 3100 Kcal |
| Soybean meal                | 26.90 | Crude Protein                 | 18.08 |
| Corn Gluten Meal            | 4.00  | Lysine                        | 1.04  |
| Soybean oil                 | 3.10  | Methionine                    | 0.49  |
| DL-Methionine               | 0.18  | Threonine                      | 0.74  |
| L-Lysine sulphate           | 0.40  | Tryptophan                    | 0.24  |
| Sodium chloride             | 0.30  | Calcium                        | 0.86  |
| Choline chloride (50%)      | 0.15  | Available Phosphorus          | 0.32  |
| Vitamin premix^2            | 0.02  | Met+Cys^3                     | 0.80  |
| Trace mineral premix^1      | 0.20  |                               |       |
| Dicalcium phosphate         | 1.40  |                               |       |
| Limestone                   | 1.20  |                               |       |
| Phytase                     | 0.02  |                               |       |
| Antioxidant                 | 0.03  |                               |       |
| Medical stone               | 0.05  |                               |       |

1 The trace mineral premix provided the following per kg of diets: Cu, 16 mg (as CuSO_4·5H_2O); Zn, 110 mg (as ZnSO_4); Fe, 80 mg (as FeSO_4·H_2O); Mn, 120 mg (as MnO); Se, 0.3 mg (as Na_2SeO_3); I, 1.5 mg (as KI); Co, 0.5mg.

2 The vitamin premix provided the following per kg of diets: vitamin A, 10,000 IU; vitamin D3, 2,400 IU; vitamin E, 20 mg; vitamin K3, 2 mg; vitamin B1, 2 mg; vitamin B2, 6.4 mg; VB6, 3 mg; VB12, 0.02 mg; biotin, 0.1 mg; folic acid, 1 mg; pantothentic acid, 10 mg; nicotinamide, 30 mg.

3 Met+Cys: Methionine+ Cysteine
| Gene  | Primer sequence (5’-3’) | Size | Accession NO. |
|-------|-------------------------|------|---------------|
| GAPDH | Forward: GGTAGTGAAGGCTGCTGCTGATG  
Reverse: AGTTCCACAACACGGTTCGCTGATC | 200  | NM_204305.1   |
| ERBB4 | Forward: ATCACCAGCATCGAGCACAACAG  
Reverse: CAGGTTCTCCAGTGGCAGGTATTC | 114  | NM_001030365.1|
| TMOD4 | Forward: GATGGAGATGGGACGAGATGCTG  
Reverse: CCTTCTCGTGCGACGAGGATTC | 135  | NM_204774.1   |
| PTGS2 | Forward: ACTGCTGGCCTCGGTCCCTTG  
Reverse: CCTCGTGCACTTCACTACCG | 121  | NM_001167719.1|
| COL1A2| Forward: TCCTCCTGTAACAACGGTCCTG  
Reverse: GAGACCATTGCAGCACTTACC | 85   | NM_001079714.2|
| POSTN | Forward: CAGCCGCATCTGTCACATGAC  
Reverse: CTTCATGTAGCCAGGACCTC | 200  | NM_001030541.1|
| COL14A1| Forward: CCAACTGAGCAGCAGACTC  
Reverse: TCCACTAGGAACACCAGGAGC1TCC | 107  | NM_205334.1   |
| TGBF1 | Forward: ACCACCAGGACAGCAGACTC  
Reverse: GTTGAGGTCAAGACAGCAGAGC | 87   | NM_205036.1   |
| ACTN1 | Forward: GCGTGGAACAGATTGCTGCTATTG  
Reverse: ATCTTCTGGCCACCTGGGCATTGAC | 88   | NM_204127.1   |
| NDUFA2| Forward: CACGAGCAGACGACTC  
Reverse: TTGGCAACTTCATCCAGCAGCAGG | 159  | NM_001302137.1|
| ADAM19| Forward: GACAGGAGAGGACGACTGACCTGAC  
Reverse: AGGAAGCAGGCTCAGGACTGACTG | 166  | NM_001195122.1|
| CCR5 | Forward: GAGATCGCTGTGCGAGGATTC  
Reverse: TGCTGGTGAAGGATGCTAGG | 159  | NM_001271141.1|

Table 3. Enzyme activities of the breast muscle.

| Enzyme | L      | H      | COMB   | SEM    | P-value |
|--------|--------|--------|--------|--------|---------|
| CK     | 2.51   | 2.41   | 2.25   | 0.12   | 0.702   |
| LDH    | 450.38a| 724.10b| 383.22a| 56.74  | 0.022   |
| T-AOC  | 100.81 | 82.17  | 86.01  | 8.25   | 0.650   |
| MDH    | 1.37   | 1.21   | 1.53   | 0.08   | 0.252   |
| Anti-superoxide anion | 10.30 | 9.32 | 10.39 | 0.39 | 0.489 |
| Hydroxyproline | 155.56 | 164.22 | 172.01 | 8.51 | 0.755 |

Table 4. Muscle contraction and cell location related pathways
| GO ID       | Term Type | Description                           | P-value   | Genes                      |
|-------------|-----------|---------------------------------------|-----------|----------------------------|
| GO:0044449  | CC        | contractile fiber part                | 0.026498  | NOS1; TMOD4                |
| GO:0006936  | BP        | muscle contraction                    | 0.000194  | MYLK2; NOS1                |
| GO:0006941  | BP        | striated muscle contraction           | 0.000908  | MYLK2; NOS1                |
| GO:0003012  | BP        | muscle system process                 | 0.00051   | MYLK2; NOS1                |
| GO:0051015  | MF        | actin filament binding                 | 0.002704  | TMOD4                      |
| GO:0003779  | MF        | actin binding                         | 0.000614  | TMOD4                      |
| GO:0008092  | MF        | cytoskeletal protein binding          | 0.033316  | TMOD4                      |
| GO:0004687  | MF        | myosin light chain kinase activity    | 0.022364  | MYLK2                      |

**Table 5.** Ion transport related pathways

| GO ID       | Term Type | Description                           | P-value   | Genes                      |
|-------------|-----------|---------------------------------------|-----------|----------------------------|
| GO:0030001  | BP        | metal ion transport                   | 0.015075  | KCNJ12                     |
| GO:0002028  | BP        | regulation of sodium ion transport    | 0.017458  | NOS1                       |
| GO:0051365  | BP        | cellular response to potassium ion starvation | 0.011244 | SLC38A3                    |
| GO:0006813  | BP        | potassium ion transport               | 0.030866  | KCNJ12                     |
| GO:0034220  | BP        | ion transmembrane transport           | 0.015681  | SLC38A4; SLC38A3; KCNJ12   |
| GO:0010107  | BP        | potassium ion import                 | 0.004526  | KCNJ12                     |
| GO:0006813  | BP        | potassium ion transport               | 0.030866  | KCNJ12                     |
| GO:0098655  | BP        | cation transmembrane transport        | 0.024337  | SLC38A3; KCNJ12            |
| GO:0006812  | BP        | cation transport                      | 0.027707  | SLC38A3; KCNJ12            |
| GO:0098662  | BP        | inorganic cation transmembrane transport | 0.046453 | KCNJ12                     |
| GO:0015075  | MF        | ion transmembrane transporter activity | 0.008902 | KCNA7; SLC38A4; SLC38A3     |
| GO:0046873  | MF        | metal ion transmembrane transporter activity | 0.007993 | KCNJ12                     |
| GO:0008324  | MF        | cation transmembrane transporter activity | 0.01451  | SLC38A3; KCNJ12            |
| GO:0022890  | MF        | inorganic cation transmembrane transporter activity | 0.022537 | KCNJ12                     |
| GO:0005261  | MF        | cation channel activity               | 0.045897  | KCNJ12                     |
| GO:0005216  | MF        | ion channel activity                  | 0.03925   | KCNA7; KCNJ12              |
| GO:0015276  | MF        | ligand-gated ion channel activity     | 0.026498  | KCNJ12                     |
| GO:0015079  | MF        | potassium ion transmembrane transporter activity | 0.029581 | KCNJ12                     |
### Table 7. Proteolysis, immune and stress related pathways

| GO ID       | Term Type | Description                              | P-value   | Genes                  |
|-------------|-----------|------------------------------------------|-----------|------------------------|
| GO:0008234  | MF        | cysteine-type peptidase activity         | 0.032179  | TINAG; USP24; OTUD1    |
| GO:0031463  | CC        | Cul3-RING ubiquitin ligase complex        | 0.028791  | KEAP1; KLHL34          |
| GO:0010499  | BP        | proteasomal ubiquitin-independent protein catabolic process | 0.03336   | KEAP1                  |
| GO:0010508  | BP        | positive regulation of autophagy          | 0.034688  | SMCR8                  |
| GO:1902902  | BP        | negative regulation of autophagosome assembly | 0.03336   | SMCR8                  |
| GO:1901096  | BP        | regulation of autophagosome maturation    | 0.011244  | SMCR8                  |
| GO:1901098  | BP        | positive regulation of autophagosome maturation | 0.011244  | SMCR8                  |

### Immune and stress related pathways

| GO ID       | Term Type | Description                              | P-value   | Genes                  |
|-------------|-----------|------------------------------------------|-----------|------------------------|
| GO:0031867  | MF        | EP4 subtype prostaglandin E2 receptor binding | 0.005638  | FEM1A                  |
| GO:0031862  | MF        | prostanoid receptor binding               | 0.005638  | FEM1A                  |
| GO:0050691  | BP        | regulation of defense response to virus by host | 0.031097  | ALKBH5; ALPK1          |
| GO:0022230  | BP        | positive regulation of defense response to virus by host | 0.026558  | ALKBH5; ALPK1          |
| GO:0071214  | BP        | cellular response to abiotic stimulus    | 0.042948  | CDKN1A; SLC38A3        |
| GO:0009991  | BP        | response to extracellular stimulus       | 0.022488  | ACACB; CDKN1A; SLC38A3 |
| GO:0031667  | BP        | response to nutrient levels               | 0.018345  | ACACB; CDKN1A; SLC38A3 |

### Table 8. Muscle development related pathway.
Table 9. Cellular matrix and collagen related pathway.

| GO ID     | Term Type | Description                                      | P-value    | Genes                      |
|-----------|-----------|--------------------------------------------------|------------|----------------------------|
| GO:0030198BP   | extracellular matrix organization                 | 1.05E-06  | MMP9; TGFBI; ABI3BP; POSTN; FBLN1, etc |
| GO:0044420CC   | extracellular matrix component                    | 2.83E-05  | COL1A2; FN1; THBS2; THBS4; LTBP1, etc |
| GO:0005614CC   | interstitial matrix                                | 0.013106  | FN1; ABI3BP                 |
| GO:0043062BP   | extracellular structure organization               | 1.15E-06  | MMP9; TGFBI; ABI3BP; ADAMTS14; COL12A1, etc |
| GO:0005201MF   | extracellular matrix structural constituent        | 0.017449  | MGP; VCAN; FBLN1            |
| GO:0019897CC   | extrinsic component of plasma membrane             | 0.000874  | SERPINE2                   |
| GO:1990430MF   | extracellular matrix protein binding               | 0.047928  | ITGGB8                     |

Table 10. Cell adhesion and antioxidant related pathway.

| GO ID     | Term Type | Description                                      | P-value    | Genes                      |
|-----------|-----------|--------------------------------------------------|------------|----------------------------|
| GO:0032964BP   | collagen biosynthetic process                     | 0.047928  | ADAMTS3                   |
| GO:0032963BP   | collagen metabolic process                         | 0.027114  | MMP9; ADAMTS3             |
| GO:0010712BP   | regulation of collagen metabolic process           | 0.023978  | SERPINF2; FAP             |
| GO:0010710BP   | regulation of collagen catabolic process           | 0.032211  | FAP                       |
| GO:0030199BP   | collagen fibril organization                       | 0.000216  | ADAMTS14; SFRP2; LUM; SERPINF2 |
| GO:005518MF    | collagen binding                                  | 0.00265   | TGFB1; ABI3BP; COMP; LUM  |
| GO:005540MF    | hyaluronic acid binding                           | 0.037432  | TNFAIP6; VCAN             |
| GO:005581MC    | collagen trimer                                   | 0.000699  | COL1A2; COL12A1; COLEC12C; LUM; COL14A1 |
| GO:005583CC    | fibrillar collagen trimer                          | 0.001541  | COL1A2; LUM               |
| GO:005539MF    | glycosaminoglycan binding                         | 3.59E-09  | MDK; SLIT3; NOV; SERPINE2; JCHAIN, etc |
| GO:1901617BP   | organic hydroxy compound biosynthetic process      | 0.033592  | NR4A2; PLTP; LCAT; AKR1D1 |
| GO ID     | Term Type | Description                                                                 | P-value  | Genes                                                                 |
|----------|-----------|-----------------------------------------------------------------------------|----------|-----------------------------------------------------------------------|
| GO:0007155 | BP        | cell adhesion                                                               | 3.1E-08  | DSG2; TGFBI; NOV; FN1; THBS2; COMP, etc                                |
| GO:0098609 | BP        | cell-cell adhesion                                                          | 0.030671 | DSG2; CSTA; NOV; CDH11; THBS4; BMP5, etc                              |
| GO:0007160 | BP        | cell-matrix adhesion                                                        | 0.02187  | FN1; ITGB8; ITG8                                                     |
| GO:0050839 | MF        | cell adhesion molecule binding                                              | 0.000141 | DSG2; THY1; TGFBI; NOV; FN1; THBS4, etc                               |
| GO:005911  | CC        | cell-cell junction                                                          | 0.001091 | DSG2; CD3E; GJA1, NOV; ABCB11; ACTN1, etc                            |
| GO:0007045 | BP        | cell-substrate adherens junction assembly                                   | 0.010838 | THY1; FN1                                                            |
| GO:0045216 | BP        | cell-cell junction organization                                             | 2.94E-06 | DSG2; THY1; GJA1; FN1; WNT11; FSCN1                                    |
| GO:0007043 | BP        | cell-cell junction assembly                                                 | 0.028627 | WNT11; FSCN1                                                         |
| GO:0034332 | BP        | adherens junction organization                                               | 0.048995 | THY1; FN1                                                            |
| GO:0034329 | BP        | cell junction organization                                                  | 0.002273 | THY1; FN1; WNT11; FSCN1                                              |
| GO:0010811 | BP        | positive regulation of cell-substrate adhesion                              | 0.00285  | THY1; FN1; ABI3BP; EDIL3; FBLN1                                      |
| GO:0034333 | BP        | adherens junction assembly                                                  | 0.027114 | THY1; FN1                                                            |
| GO:0005178 | MF        | integrin binding                                                            | 2.56E-07 | THY1; TGFBI; NOV; FN1; THBS4; EDIL3, etc                             |

**Antioxidant related pathways**

| GO ID     | Term Type | Description                                                                 | P-value  | Genes                                                                 |
|----------|-----------|-----------------------------------------------------------------------------|----------|-----------------------------------------------------------------------|
| GO:0016209 | MF        | antioxidant activity                                                        | 0.040595 | MGST2; PTGS2; SOD3                                                     |
| GO:0004784 | MF        | superoxide dismutase activity                                                | 0.047928 | SOD3                                                                  |
| GO:0006801 | BP        | superoxide metabolic process                                                 | 0.00047  | NCF1; SOD3; CYBB                                                       |
| GO:1901031 | BP        | regulation of response to reactive oxygen species                           | 0.048995 | HGF                                                                   |
| GO:0050664 | MF        | oxidoreductase activity, acting on NAD(P)H, oxygen as acceptor              | 0.01556  | NCF1; CYBB                                                            |
| GO:0098869 | BP        | cellular oxidant detoxification                                              | 0.040595 | MGST2; PTGS2; SOD3                                                     |

**Table 11. Muscle development and mitochondria related pathway.**

| Number | GO ID     | Term Type | Description                                                                 | P-value  | Genes                                                                 |
|--------|-----------|-----------|-----------------------------------------------------------------------------|----------|-----------------------------------------------------------------------|
| Muscle development related pathways |
| GO:1901741  | BP        | positive regulation of myoblast fusion                                     | 0.000717 | MYF6                                                                  |
| GO:0010831  | BP        | positive regulation of myotube differentiation                             | 0.003478 | MYF6                                                                  |
| GO:0014743  | BP        | regulation of muscle hypertrophy                                           | 0.00447  | LMCD1:TRPC3                                                           |
| GO:0048643  | BP        | positive regulation of skeletal muscle tissue development                  | 0.00447  | MYF6                                                                  |
| GO:1901863  | BP        | positive regulation of muscle tissue development                           | 0.020276 | MYF6                                                                  |
| GO:0051149  | BP        | positive regulation of muscle cell differentiation                         | 0.025719 | MYF6                                                                  |
| GO:0048743  | BP        | positive regulation of skeletal muscle fiber development                   | 0.035113 | MYF6                                                                  |
| GO:0045844  | BP        | positive regulation of striated muscle tissue development                  | 0.017276 | MYF6                                                                  |
| GO:0051155  | BP        | positive regulation of striated muscle cell differentiation                | 0.011102 | MYF6                                                                  |
| GO:0048636  | BP        | positive regulation of muscle organ development                            | 0.017276 | MYF6                                                                  |
| GO:0014744  | BP        | positive regulation of muscle adaptation                                   | 0.023546 | TRPC3                                                                 |
| Mitochondria related pathways |
| GO:0042775  | BP        | mitochondrial ATP synthesis coupled electron transport                      | 0.023546 | NDUFV1                                                                |
| GO:0022904  | BP        | respiratory electron transport chain                                        | 0.025719 | NDUFV1                                                                |
| GO:0022900  | BP        | electron transport chain                                                   | 0.029237 | NDUFV1                                                                |
| GO:0098800  | CC        | respiratory chain complex                                                  | 0.002546 | NDUFV1:NDUF5:NDUF2                                                    |
| GO:0045271  | CC        | respiratory chain complex I                                                | 0.000532 | NDUFV1:NDUF5:NDUF2                                                    |
| GO:0005747  | CC        | mitochondrial respiratory chain complex I                                  | 0.00532  | NDUFV1:NDUF5:NDUF2                                                    |
| GO:0098798  | CC        | mitochondrial protein complex                                               | 0.022727 | TOMM6:NDUFV1:NDUF5:NDUF2                                             |
| GO:0098800  | CC        | inner mitochondrial membrane protein complex                               | 0.009305 | NDUFV1:NDUF5:NDUF2                                                    |
| GO:0005742  | CC        | mitochondrial outer membrane translocase complex                           | 0.046544 | TOMM6                                                                 |
| GO:0098779  | BP        | mitophagy in response to mitochondrial depolarization                      | 0.04939  | LMCD1:ZN5F93                                                           |
Table 12. Hyaluronan and redox related pathway.

| Number | GO ID     | Term Type | Description                              | P-value     | Genes             |
|--------|-----------|-----------|------------------------------------------|-------------|-------------------|
|        | GO:0030213| BP        | hyaluronan biosynthetic process           | 0.023546    | HYAL1             |
|        | GO:0030214| BP        | hyaluronan catabolic process              | 0.000207    | HYAL3|HYAL1 |
|        | GO:0030212| BP        | hyaluronan metabolic process              | 0.001519    | HYAL3|HYAL1 |
|        | GO:1900106| BP        | positive regulation of hyaluronan cable assembly | 0.017711   | HYAL1         |
|        | GO:0004415| MF        | hyaluronoglucosaminidase activity         | 0.000514    | HYAL3|HYAL1 |
|        | GO:0033906| MF        | hyaluronoglucuronidase activity           | 0.011842    | HYAL3         |
|        | GO:0036117| CC        | hyaluronan cable                          | 0.011842    | HYAL1          |
|        | GO:0005001| MF        | hyaluronan synthase activity              | 0.017711    | HYAL1          |
|        | GO:0006027| BP        | glycosaminoglycan catabolic process       | 0.00122     | HYAL3|HYAL1 |
|        | GO:0030203| BP        | glycosaminoglycan metabolic process       | 0.039481    | HYAL3|HYAL1 |
|        | GO:0006026| BP        | aminoglycan catabolic process             | 0.003025    | HYAL3|HYAL1 |
|        | GO:1903510| BP        | mucopolysaccharide metabolic process      | 0.013602    | HYAL3|HYAL1 |

Table 13. Immune response and inflammatory response related pathways.

| Number | GO ID     | Term Type | Description                                                                 | P-value     | Genes             |
|--------|-----------|-----------|-----------------------------------------------------------------------------|-------------|-------------------|
|        | GO:0006954| BP        | inflammatory response                                                       | 0.004612    | CCR5|ALOX5 |
|        | GO:0002532| BP        | production of molecular mediator involved in inflammatory response          | 0.01346     | ALOX5          |
|        | GO:002538 | BP        | arachidonic acid metabolite production involved in inflammatory response    | 0.008097    | ALOX5          |
|        | GO:0050778| BP        | leukotriene production involved in inflammatory response                    | 0.008097    | ALOX5          |
|        | GO:005087 | BP        | positive regulation of immune response                                        | 0.017271    | C1S|BLK |
|        | GO:0056956| BP        | complement activation                                                        | 0.005145    | C1S|BLK |
|        | GO:001867 | BP        | complement activation, lectin pathway                                        | 7.08E-05    | C1S         |
|        | GO:0004950| MF        | chemokine receptor activity                                                  | 0.047636    | CCR5         |
|        | GO:0016493| MF        | C-C chemokine receptor activity                                              | 0.02101     | CCR5         |
|        | GO:0090026| BP        | positive regulation of monocyte chemotaxis                                  | 0.02101     | CCR5         |
|        | GO:0002495| BP        | antigen processing and presentation of peptide antigen via MHC class II     | 0.032008    | MARCH1        |
|        | GO:0042287| MF        | MHC protein binding                                                          | 0.045049    | MARCH1        |

Table 14. Organic acid, fatty acid metabolic process, glycolytic and carbohydrate metabolism related pathways.
| Number | GO ID   | Term Type | Description                                      | P-value     | Genes                                      |
|--------|---------|-----------|--------------------------------------------------|-------------|--------------------------------------------|
|        | GO:0006082 BP | organic acid metabolic process | organic acid metabolic process                  | 0.002344    | PSAT1;SCD;MAT1A;ALOX5;ST3GAL1;ALDOB       |
|        | GO:0016053 BP | organic acid biosynthetic process | organic acid biosynthetic process               | 0.006961    | PSAT1;SCD;ALOX5                           |
|        | GO:0043436 BP | organic acid metabolic process | organic acid metabolic process                  | 0.002254    | PSAT1;SCD;MAT1A;ALOX5;ST3GAL1;ALDOB       |
|        | GO:0046394 BP | carboxylic acid biosynthetic process | carboxylic acid metabolic process               | 0.006961    | PSAT1;SCD;ALOX5                           |
|        | GO:0019752 BP | carboxylic acid metabolic process | carboxylic acid metabolic process               | 0.001555    | PSAT1;SCD;MAT1A;ALOX5;ST3GAL1;ALDOB       |
|        | GO:0006633 BP | fatty acid biosynthetic process | fatty acid biosynthetic process                | 0.012538    | SCD;ALOX5                                 |
|        | GO:0006636 BP | unsaturated fatty acid biosynthetic process | unsaturated fatty acid biosynthetic process    | 0.002054    | SCD;ALOX5                                 |
|        | GO:0016215 MF | acyl-CoA desaturase activity | acyl-CoA desaturase activity                   | 0.002706    | SCD                                       |
|        | GO:0030388 BP | fructose 1,6-bisphosphate metabolic process | fructose 1,6-bisphosphate metabolic process    | 0.01613     | ALDOB                                     |
|        | GO:006000 BP | fructose metabolic process | fructose metabolic process                     | 0.018794    | ALDOB                                     |
|        | GO:0070061 MF | fructose binding | fructose binding                               | 0.010782    | ALDOB                                     |
|        | GO:0061609 MF | fructose-1-phosphate aldolase activity | fructose-1-phosphate aldolase activity         | 0.002706    | ALDOB                                     |
|        | GO:004332 MF | fructose-bisphosphate aldolase activity | fructose-bisphosphate aldolase activity        | 0.010782    | ALDOB                                     |
|        | GO:0005975 BP | carbohydrate metabolic process | carbohydrate metabolic process                 | 0.029095    | GALNT16;ST3GAL1;ALDOB                     |
|        | GO:0030246 MF | carbohydrate binding | carbohydrate binding                           | 0.041651    | GALNT16;ALDOB                              |

**Additional File Legends**

**Fig. S1 Inflammatory mediator regulation of RTP channels pathway analysis.** Differential expressed genes that are involved in the inflammatory mediator regulation of RTP channels [map04750], are highlighted. We thank Kanehisa Laboratories for providing the copyright permission of KEGG pathway maps [75].

**Fig. S2 Chemokine signaling pathway analysis.** Differential expressed genes that are involved in the chemokine signaling pathway [map04062], are highlighted. We thank Kanehisa Laboratories for providing the copyright permission of KEGG pathway maps [75].

**Fig. S3 Calcium signaling pathway analysis.** Differential expressed genes that are involved in the calcium signaling pathway [map04020], are highlighted. We thank Kanehisa Laboratories for providing the copyright permission of KEGG pathway maps [75].

**Fig. S4 ECM-receptor interaction pathway analysis.** Differential expressed genes that are involved in the inflammatory mediator regulation of RTP channels [map04512], are highlighted. We thank Kanehisa Laboratories for providing the copyright permission of KEGG pathway maps [75].

**Fig. S5 Adherens junction pathway analysis.** Differential expressed genes that are involved in the adherens junction [map04520], are highlighted. We thank Kanehisa Laboratories for providing the copyright permission of KEGG pathway maps [75].

**Fig. S6 Focal adhesion pathway analysis.** Differential expressed genes that are involved in the focal adhesion [map04510], are highlighted. We thank Kanehisa Laboratories for providing the copyright permission of KEGG pathway maps [75].

**Fig. S7 Oxidative phosphorylation pathway analysis.** Differential expressed genes that are involved in the oxidative phosphorylation [map00190], are highlighted. We thank Kanehisa Laboratories for providing the copyright permission of KEGG pathway maps [75].

**Fig. S8 Regulation of lipolysis in adipocytes pathway analysis.** Differential expressed genes that are involved in the Regulation of lipolysis in adipocytes [map04923], are highlighted. We thank Kanehisa Laboratories for providing the copyright permission of KEGG pathway maps [75].

**Fig. S9 Glycolysis/Gluconeogenesis pathway analysis.** Differential expressed genes that are involved in the Glycolysis/Gluconeogenesis [map00010], are highlighted. We thank Kanehisa Laboratories for providing the copyright permission of KEGG pathway maps [75].

**Fig. S10 Arachidonic acid metabolism pathway analysis.** Differential expressed genes that are involved in the arachidonic acid metabolism [map00590], are highlighted. We thank Kanehisa Laboratories for providing the copyright permission of KEGG pathway maps [75].

**Figures**
Figure 1

Water holding capacity of breast muscle. Data are shown as the means ± SEM. Different letters a, b indicate that there are significant differences (P < 0.05) among these groups. L, low stocking density (14 birds/m²); H, high stocking density (18 birds/m²); COMB, combination of NAM and BA (18 birds/m²).

Figure 2

The pH values of breast muscle. Data are shown as the means ± SEM. Different letters a, b indicate that there are significant differences (P < 0.05) among these groups. L, low stocking density (14 birds/m²); H, high stocking density (18 birds/m²); COMB, combination of NAM and BA (18 birds/m²).
Figure 3
Principal Component Analysis (PCA) and Wayne (VEEN) analysis of gene sets. For the PCA graph, the distance between each sample point represents the distance of the sample. The closer the distance means higher the similarity between samples; for the VEEN graph, the numbers inside the circle represents the sum of the number of expressed genes in the group. The crossover region represents the number of consensus expressed genes for each groups.

Figure 4
Volcanic map of differential expression genes. The abscissa is the fold change of the gene expression difference between the two samples and the ordinate is the statistical test value of the gene expression. Each dot in the figure represents a specific gene, the red dot indicates a significantly up-regulated gene, the green dot indicates a significantly down-regulated gene, and the grey dot is a non-significant differential gene.

**Figure 5**

The Veen diagram and the map of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis of gene sets. For VEEEN diagram: the sum of all the numbers inside the circle represents the total gene of the set. The number, circle intersection area represents the number of shared genes among the gene sets. For the map of KEGG metabolic pathway, the red represents the pathway of the common annotation of the genes in the gene sets of two groups. We thank Kanehisa Laboratories for providing the copyright permission of KEGG pathway maps [75].
Figure 6

GO enrichment analysis of up-regulated genes in the H group. The abscissa indicates the GO term, and the ordinate indicates the enrichment ratio. "*" means $P < 0.05$, "**" means $P < 0.01$ and "***" means $P < 0.001$. 
Figure 7

GO enrichment analysis of down-regulated genes in the H group. The abscissa indicates the GO term, and the ordinate indicates the enrichment ratio. "*" means $P < 0.05$, "**" means $P < 0.01$ and "***" means $P < 0.001$. 
Figure 8

GO enrichment analysis of up-regulated genes in the COMB group. The abscissa indicates the GO term, and the ordinate indicates the enrichment ratio. "*" means $P < 0.05$, "**" means $P < 0.01$ and "***" means $P < 0.001$. 
Figure 9

GO enrichment analysis of down-regulated genes in the COMB group. The abscissa indicates the GO term, and the ordinate indicates the enrichment ratio. "*" means $P < 0.05$, "**" means $P < 0.01$ and "***" means $P < 0.001$. 
Figure 10

The mRNA relative expression of DEGs quantified by quantitative reverse transcription-PCR. Data presented as means ± SEM.
Figure 11

The graphic description of the normalization effect of nicotinamide and sodium butyrate on breast muscle.

Supplementary Files

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