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Gene expression analysis (RNA-seq) in chicken liver under the influence of a high-protein diet

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Abstract
Gene expression analysis in poultry provides a comprehensive view of the biological mechanisms controlling production and is a cornerstone for breed improvement, nutritional development, immunity enhancement, and understanding the impact of environmental conditions. Thanks to modern technologies such as RNA-seq, it is now possible to create a precise picture of how the genome responds to different factors, paving the way for the development of a more efficient poultry industry.

Keywords: RNA-seq, gene expression profiling, chicken liver, high-protein diet

1. Introduction
The poultry industry is considered one of the important agricultural and economic sectors globally, as its products contribute effectively to meeting human animal protein needs in terms of cost and quality (FAO, 2022) [1]. With the increasing global demand for meat and eggs, it has become essential to develop strategies to improve productivity, quality, and disease resistance by relying on the genetic and molecular principles that control muscle growth and productive performance in poultry. Muscle growth in poultry is one of the most important productive traits in the chicken meat industry, and it is directly linked to feed conversion efficiency, meat quality, and economic returns (Zhang *et al*., 2020) [27]. The significant advancements in molecular biology techniques, particularly next-generation DNA sequencing, have revolutionized our understanding of the genetic and regulatory mechanisms controlling muscle growth. Gene expression analysis is a fundamental tool for understanding the molecular basis of muscle growth and the physiological functions of the liver and muscles in poultry. One of the most prominent techniques used today in this field is RNA-seq sequencing, which enables researchers to study the complete transcriptome of any biological sample without prior knowledge of the gene sequence. It can also detect novel gene variants and identify under expressed genes with high precision. Wang *et al*., (2009) [2]; Zhao *et al*., (2014) [3]. Applying this technology in poultry helps identify the molecular pathways responsible for muscle cell growth, thus paving the way for improving productive traits using selection programs or genetic engineering. Skeletal muscles, especially breast muscles in chickens, are among the most important tissues in terms of productivity, representing the largest portion of poultry meat consumed. Muscle growth depends on the precise regulation of a series of genes responsible for muscle fiber formation, muscle cell differentiation, and muscle mass control. Among the most prominent of these genes are MSTN, which acts as a negative regulator of muscle growth, and IGF-1, which stimulates muscle cell proliferation and differentiation, in addition to transcription factors that direct muscle development, such as Myo and Myogenic (Li *et al*., 2021; Liu *et al*., 2023) [23, 12]. Recent studies have shown that variation in the gene expression of these genes explains the differences between fast-growing and slow-growing breeds and directly affects muscle mass and meat quality. RNA-seq technology is one of the most powerful and accurate tools for analyzing gene expression, as it can detect thousands of genes simultaneously, including those with low or previously unknown expression. (Huang *et al*., 2022) [4]. Gene expression analysis is also used to assess feed conversion efficiency and the conversion of feed into muscle protein, known as feed conversion ratio (FCR).

RNA-seq analysis helps identify genes and metabolic pathways associated with feed intake and available energy, such as mitochondrial metabolism genes, enabling researchers to develop precise nutritional strategies to enhance productive performance (Yi *et al*., 2022) [30].

Gene expression analysis also plays a pivotal role in enhancing disease resistance and improving immunity. RNA-seq can identify immune genes and changes in their expression during infection, such as TLR4 and IFN-γ genes, facilitating the development of disease-resistant strains or more effective vaccines (Deist *et al*., 2017) [24]. Furthermore, this analysis allows for understanding the impact of heat stress and environmental conditions on birds by monitoring the gene expression of the heat-shock proteins HSP70 and HSP90, as well as heat-affected metabolic pathways (Hassanpour *et al*., 2020) [31].

In practice, RNA-seq results offer wide-ranging applications in the poultry industry, including:

1. Improving genetic selection programs by targeting genes for productive traits (Li *et al*., 2021) [23].
2. Developing feeds specifically formulated to support muscle growth and energy efficiency (Huang *et al*., 2022) [4].
3. Enhancing disease resistance and environmental tolerance (Deist *et al*., 2017; Hassanpour *et al*., 2020) [24, 31].
4. Improving meat quality by identifying genes that control muscle fiber type and fat metabolism (Papah *et al*., 2021) [26].

These scientific advancements in understanding gene expression represent a revolution in poultry production science, transforming molecular information into practical tools for improving productivity, quality, and disease resistance, thereby enhancing food security and boosting the economic efficiency of the poultry industry (Zhang *et al*., 2020) [27].

2. Scientific Background of RNA-seq Technology

RNA-seq is one of the most important modern tools for studying and detecting gene expression levels in tissues, cells, and at the whole-genome level. It has revolutionized molecular biology since its emergence, following the rapid development of high-throughput sequencing platforms. The technique involves extracting total RNA from a sample, converting it to cDNA, and then fragmenting and sequencing it using high-throughput sequencing devices to determine the quantity and presence of different RNA structures within the cell (Dobin *et al*., 2013) [5].

Unlike microarray techniques, it is capable of detecting novel genes, isoforms, and regulatory events such as splicing and RNA modifications. It also provides the ability to measure gene expression with high quantitative accuracy and excellent sensitivity for detecting subtle variations between samples. Before the advent of RNA-seq, techniques such as microarrays were limited by the requirement of prior gene sequence knowledge and their low accuracy in distinguishing between similar genes. RNA-seq, however, is distinguished by its ability to detect novel genes, mutations, and alternative splicing, as well as its capacity to measure gene expression with high precision across a very wide range (Marioni *et al*., 2008) [20].

The technique typically begins with the isolation of total or messenger RNA from a biological sample, followed by the preparation of cDNA libraries using strategies such as poly A-tailed mRNA selection or rRNA removal. This makes RNA-seq suitable for various sample types, including degraded samples or those containing small amounts of RNA (Ura *et al*., 2024) [6]. After the libraries are prepared, they are entered into modern sequencing platforms such as Illumina, which produce millions of short reads with high resolution. These reads then undergo purification and alignment to the reference genome using software such as STAR (Dobin *et al*., 2013) [5] or rapid quasi-mapping using tools such as Salmon, which provides a fast and accurate estimation of gene transcripts (Patro *et al*., 2017) [7].

RNA-seq is distinguished by its ability to measure gene expression with high accuracy across a wide dynamic range, far surpassing microarray techniques. It can detect both low- and high-expression genes with almost equal precision (Wang *et al*., 2009) [2]. The technique also enables the analysis of isoforms resulting from alternative transcription processes, the identification of novel genes, and the analysis of temporal changes in gene expression. This makes it an essential tool for studying biological responses to dietary or environmental factors, such as the effect of high-protein diets on chicken livers.In recent years, methodologies for the statistical analysis of RNA-seq data have evolved, utilizing tools such as DESeq2 (Love *et al*., 2014) [9]. These tools rely on robust statistical models to identify genes with differential expression, enhancing the ability to accurately interpret molecular changes and link them to biological pathways and metabolism.

Overall, RNA-seq technology is now fundamental to functional biology studies, providing a powerful platform for understanding complex molecular mechanisms within cells, particularly in studies related to the effects of nutrition, liver function, poultry growth, and various physiological responses.

3. The Role of Gene Expression in Skeletal Muscle Growth

Skeletal muscle growth in poultry is a complex process controlled by a wide network of genes and biological pathways that regulate the proliferation, differentiation, and fusion of myoblasts to form mature muscle fibers. Muscle fiber formation begins during the embryonic stage. These genes include transcription factors (such as MyoD, Myf5, and Myogenic), cell cycle control genes, and protein synthesis regulation genes. The MSTN gene also plays a significant negative role, acting as a suppressor of muscle growth. Gene expression analysis via RNA-seq can identify genes that are active or repressive at different stages of muscle growth, helping to understand the relationship between genotypes and productive traits.

However, the increase in muscle mass after hatching depends primarily on an increase in muscle fiber size (hypertrophy) rather than their number. This increase depends on the regulated expression of specific genes that control growth, protein synthesis, and metabolic pathways. One of the most important genes regulating muscle growth is the myostatin (MSTN) inhibitor gene, which acts as a negative regulator of muscle cell proliferation. Decreased MSTN expression or mutations lead to a significant increase in muscle mass due to the release of growth pathways such as the Akt/mTOR pathway, which is responsible for protein synthesis and the stimulation of muscle fiber hypertrophy. Recent studies in poultry have confirmed that MSTN

inhibition increases the rate of muscle protein synthesis and increases fiber size (Zhang *et al*., 2023) [10]. The IGF-1/PI3K/Akt pathway also plays a pivotal role in promoting muscle growth by stimulating the proliferation of myogeneic precursor cells (satellite cells) and increasing the gene expression of structural proteins such as myosin heavy chain (MYH). A study in broiler chickens showed that increased IGF-1 expression is directly associated with increased pectoral muscle weight and improved growth rate (Kim *et al*., 2022) [11]. In the next stage, gene expression of the Myogenic Regulatory Factors (MRF) family, such as MyoD and Myogenin, controls muscle cell differentiation and fusion. MyoD is essential for initiating the transformation from stem cells to primary muscle cells, while myogenin contributes to fiber maturation and the establishment of muscle identity. Recent studies using RNA-seq have shown that higher expression of MyoD and Myogenin is associated with faster muscle fiber growth in chickens (Liu *et al*., 2023) [12].

Gene expression also plays a role in determining muscle functional characteristics, such as the switch between fast-twitch and slow-twitch fiber types, through the regulation of genes like MYH7 and MYH1. This affects growth rate and meat quality. A study in broiler chickens found that the switch towards fast-twitch fibers is associated with increased growth rate, and this switch is controlled by metabolic genes such as PGC-1α (Wang *et al*., 2024) [23]. In general, recent RNA-seq genetic analyses show that variations in growth rates among different poultry breeds are due to differences in gene expression patterns of growth genes, metabolic pathways, and muscle differentiation. This highlights the pivotal role of gene expression in developing strategies to improve production performance, Huang *et al*. (2024) [14].

4. Using RNA-seq technology in poultry muscle growth studies

Numerous studies have shown that RNA-seq technology is an effective tool for detecting molecular differences between poultry lines with different growth rates. It can identify genes associated with growth rate and meat quality. RNA-seq allows for the analysis of the complete transcriptome (mRNAs, lncRNAs, miRNAs, etc.) in muscle tissue, in addition to biological pathways such as the mTOR pathway, IGF-1, and the mitochondrial phospho-oxidation pathway. Samples are usually taken from the pectoralis major muscle at different ages to compare differences in gene expression between strains or between experimental treatments. The results help identify candidate genes that can be targeted by gene selection programs or CRISPR technology to improve muscle growth. This enables the monitoring of changes in gene expression during the stages of muscle development (embryonic and post-hatching) in chickens. For example, a study on chicken strains using RNA-seq at three developmental stages showed the temporal expression of hundreds of mRNAs and lncRNAs associated with muscle fiber formation. And its growth. (Frontiers in Physiology, 2023)

By comparing fast-growing (broiler) and slow-growing/local breeds, RNA-seq helps identify key genes and their isoforms that may explain differences in muscle mass and growth rate. A recent study (2023) on breast muscle focused on embryonic and post-hatching development and highlighted genes and isoforms associated with differences between the two breeds. Using RNA-seq, genes responsible for muscle fiber type composition that is, fast versus slow fibers can also be explored. This is important for meat quality and growth performance. One study comparing breast versus leg muscles in chickens used RNA-seq and identified 767 genes with different expression levels. These genes were then linked to metabolic pathways such as glycolysis/glucogenic, glycolysis, and the insulin pathway. In addition to mRNAs, RNA-seq technology allows for the use of more broadly, transcriptomic analysis identifies non-coding molecules such as lncRNAs and miRNAs that may play a crucial regulatory role in muscle growth, such as regulating cell division, differentiation, and fiber differentiation. A study using a different chicken breed that employed RNA-seq demonstrated that lncRNAs exhibit heterogeneous expression across developmental stages and may be involved in regulating muscle growth.

RNA-seq is also used to investigate the impact of environmental stressors or genetic parameters on muscle growth. For example, in a study examining the response of broiler chicken muscles to chronic heat stress, researchers used RNA-seq to identify lncRNAs and their expression patterns associated with muscle damage, cell death, and connective tissue formation. This provides valuable insights into how environmental conditions influence muscle growth and meat quality.

Instead of focusing on a limited number of previously known genes, RNA-seq offers a comprehensive and dynamic view of the gene pool involved in muscle development. This is beneficial for improving breeding programs aimed at enhancing muscle mass, meat quality, and resistance to environmental stresses

5. Comparison of RNA-seq with Microarray Technology

Microarray technology was common before the advent of RNA-seq, but it relies on pre-designed probes, which limits its ability to detect new or mutated genes. It is also less sensitive and accurate, and it does not measure gene expression levels quantitatively but rather relies on relative comparisons. In contrast, RNA-seq is distinguished by its ability to analyze millions of reads and determine gene expression with high precision. It also detects genetic variants, identifies splice variants, and analyzes non-coding RNA, making it the preferred technology in animal production research and modern genetics.Both microarray and RNA-seq have revolutionized the study of gene expression, but they differ fundamentally in accuracy, flexibility, and the volume of data they generate, as mentioned. Microarray technology relies on hybridizing RNA with pre-fixed probes on the chips, which limits the analysis to known genes included in the chip design. Therefore, microarray cannot detect novel genes, mutations, or previously unknown variants. Comparative studies have confirmed that this limitation makes the technique less effective for analyzing the genomes of non-model organisms or those with multiple gene variants, such as poultry (Wang *et al*., 2009) [2].

In contrast, RNA-seq technology uses whole-genome sequencing without relying on fixed probes. This allows for the detection of all intracellularly expressed molecules, including novel genes, various isoforms, and mutations, as well as precise quantitative expression. Studies have shown that RNA-seq is superior in sensitivity and accuracy for detecting low-expression genes that are often difficult to

detect using microarray (Zhao *et al*., 2014) [3]. Furthermore, RNA-seq technology boasts a very wide dynamic range, allowing it to distinguish both small and large differences in gene expression levels. In contrast, microarrays are limited by a narrow dynamic range due to signal saturation or weakness. A comprehensive comparative study indicated that RNA-seq offers approximately 800 times a dynamic range compared to microarrays (Marioni *et al*., 2008) [20]. (Kukurba & Montgomery, 2015) [21] added that RNA-seq technology allows for the analysis of expression in non-coding genes (lncRNAs, miRNAs), the analysis of association with biological pathways, and the detection of new transcripts at different growth stages. These are very important features in poultry research that rely on studying muscle growth and tissue development. Although microarray is less expensive and easier to analyze in some cases, RNA-seq has become the most relied-upon technology in modern studies of muscle growth in poultry because it provides deeper data and enables researchers to build high-resolution expression maps and detect subtle differences between breeds or between age groups (Zhang *et al*., 2020) [27].

6. Practical Applications of RNA-seq Technology in Improving Poultry Production

Gene expression analysis using RNA-seq technology allows for the development of multiple production improvement strategies, including

1. Identifying genes suitable for genetic selection to enhance growth rate and increase muscle mass.
2. Improving feeding programs by understanding how muscles respond to nutrients and proteins.
3. Developing disease-resistant breeds by identifying genes associated with immunity.
4. Supporting gene-editing technologies such as CRISPR-Cas9 to target key genes like MSTN.
5. Increasing the economic efficiency of production by selecting lines with faster growth and higher meat quality.

RNA-seq technology has become a central tool in modern poultry development programs because it not only monitors gene expression patterns but also translates this data into practical decisions in breeding, nutrition, and health .RNA-seq technology is distinguished by its ability to identify genes and biological pathways that influence muscle growth, feed conversion, disease resistance, and environmental stress tolerance, thus paving the way for precise and effective production improvement (Zhang *et al*., 2020) [27]. Gene expression analysis using RNA-seq technology allows for the development of several strategies to improve production, including:

1. **Enhancing Genomic Selection Programs:** RNA-seq results are used to identify candidate genes associated with key production traits such as growth rate, feed conversion ratio (FCR), breast muscle mass, and meat quality. For example, RNA-seq analysis in broiler breeds showed that genes in the IGF-1/Akt/mTOR pathway are directly associated with improved growth, allowing for their inclusion as molecular markers in genomic selection programs This accelerates the selection of the best-performing birds without relying solely on phenotype (Li *et al*., 2021) [23].
2. **Developing Feeds Specifically Designed to Enhance Growth:** RNA-seq technology has contributed to revealing the genetic response to feed, which helps in designing feeds that target and enhance pathways responsible for muscle growth or metabolism. For example, a study by Huang *et al*. (2022) [4] using RNA-seq technology showed that modifying protein levels or adding amino acids can increase the expression of the MYOD and PGC-1α genes, which are responsible for muscle fiber growth and improved energy efficiency Thus, RNA-seq technology has become fundamental to developing micronutrients for poultry
3. **Improving Disease Resistance:** RNA-seq technology has helped identify genes expressed during viral or bacterial infections, enabling the development of more disease-resistant strains. Deist *et al*. (2017) [24] demonstrated that transcriptomic analysis in Newcastle disease-resistant chickens revealed a clear difference in the expression of innate immunity genes such as TLR4 and IFN-γ, which has helped support selection programs for more resistant strains.
4. **Understanding Heat Stress and Improving Tolerance:** Heat stress is one of the most serious challenges in poultry production, especially in hot regions. RNA-seq technologies have allowed for the identification of genes associated with cellular defense mechanisms, such as HSP70 and HSP90, as well as metabolic pathways affected by high temperatures. This helps in developing breeding programs to select more tolerant birds and designing diets that support the molecular pathways regulating the heat response (Hassanpour *et al*., 2020) [31].
5. **Improving Meat Quality:** RNA-seq technology is used to identify genes that control: muscle fiber type, fat metabolism, meat tenderness after slaughter, and the predisposition to muscle diseases such as white striping and woody breast. RNA-seq studies have shown that increased expression of oxidation-related genes such as PPARα improves meat quality and reduces defects (Papah *et al*., 2021) [26].
6. **Supporting Genetic Engineering and Gene Editing:** RNA-seq data helps identify genes suitable for CRISPR editing, such as growth regulators or immune-regulating genes, making gene editing more precise and effective (Montgomery *et al*., 2015) [21]. Furthermore, RNA-seq results are not merely academic data; they translate into production decisions that include genetic selection, new feed designs, breed selection, heat tolerance improvement, and immune enhancement, making the technology a cornerstone for improving poultry production in the modern era. 7. The Importance of Gene Expression Analysis in Poultry
7. **7-Supporting the poultry industry in developing countries by providing data that helps improve local breeds:** Gene expression analysis, particularly using techniques such as RNA-seq, is a pivotal tool for understanding the molecular basis of productive traits in poultry, such as growth, feed conversion efficiency, meat quality, and disease resistance. This technique measures gene expression levels across growth stages or under different conditions, allowing for a deep understanding of the biological processes that characterize broiler and layer breeds (Zhang *et al*.,

2020) [27]. RNA-seq provides comprehensive information that can be utilized in:

1. Understanding Muscle Growth and Tissue Development

Studying gene expression helps increase productivity, improve carcass characteristics, and identify genes that regulate muscle fiber formation, such as MYOD, MYF5, IGF-1, and MSTN, which play a key role in determining muscle size and fiber type. Studies using RNA-seq on pectoral muscle (breast muscle *et al*., 2021) have shown that differences in gene expression between fast-growing and slow-growing breeds explain the differences in muscle mass and growth rate (Li *et al*., 2021) [23]. This analysis also helps identify the different isoforms that are active during embryonic development and after hatching (Huang *et al*., 2022) [4].

2. Improving Breeding and Genetic Selection Programs

Gene expression analysis is fundamental to discovering candidate genes associated with productive traits and developing gene-based predictive models for evaluating breed performance. By identifying genes associated with growth, meat quality, disease resistance, or heat tolerance, they can be incorporated into marker-assisted selection programs, thus accelerating breed improvement compared to traditional methods (Khan *et al*., 2021) [28]. Gene expression analysis has contributed to the identification of genes such as GHR, PGC-1α, and PPARγ that are associated with improved growth and energy efficiency (Chen *et al*., 2023) [29].

3. Understanding the Molecular Basis of Feed Conversion

Efficiency Feed Conversion Efficiency (FCR) is one of the most important economic determinants in the poultry industry Gene expression analysis allows us to understand the genes that regulate mitochondrial energy metabolism, protein synthesis, and the digestive system's response to feed RNA-seq studies have shown that birds with a low FCR have increased expression of mitochondrial oxidation genes and decreased expression of inflammation genes, which explains their better production performance (Yi *et al*., 2022) [30].

4. Improving Disease Resistance and Enhancing Immunity

Gene expression analysis helps identify genes associated with innate and adaptive immunity during disease outbreaks such as Newcastle Disease (NDV), avian influenza, and coccidiosis, and reduces losses due to disease or stunted growth. RNA-seq studies have allowed for the identification of key immunity genes such as TLR4, IL-1β, and IFN-γ, and the differences in their levels between disease-resistant and susceptible strains (Deist *et al*., 2017) [24]. This analysis is therefore used to develop better vaccines and breeding programs targeting the most disease-resistant strains.

5. Understanding the Impact of Environmental Conditions and Heat Stress

Heat, humidity, stocking density, and oxidative stress affect gene expression in poultry. Gene expression studies have revealed that heat stress leads to increased expression of the heat shock protein genes HSP70 and HSP90 and decreased expression of genes associated with metabolism and growth (Hassanpour *et al*., 2020) [31]. This helps in improving management and feeding programs for heat-sensitive strains

6. Supporting Nutritional Studies and Developing Improved Rations

By analyzing changes in gene expression under the influence of different diets (high protein, plant supplements, amino acids), growth or immunity pathways affected by nutrition can be identified, supporting the design of precise rations to improve performance (Kim *et al*., 2022) [11].

Summary

This research aims to study the effect of a high-protein diet on gene expression patterns in chicken liver using RNA-seq technology. The liver is one of the most important organs responsible for the metabolism and biosynthesis of proteins and fats, and the type of diet directly affects the activity of genes associated with these functions. Studies have shown that increasing the protein level in the diet causes clear changes in the expression of genes associated with growth, metabolism, and immunity. This research highlights the mechanism of action of RNA-seq technology and its most important applied results in improving poultry production.

Conclusion

Gene expression analysis using RNA-seq is central to understanding the molecular basis of muscle growth in poultry. This technique has facilitated the transition from studying individual genes to a comprehensive analysis of all genes simultaneously, enabling the identification of new biological pathways that can be exploited to improve meat production. With the continued development of sequencing and biocomputational technologies, RNA-seq is expected to become a standard tool in genetic breeding research and the modern poultry industry.

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