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Transcriptomic Insights Into Gene Expression Differences Between High And Low Milk Producing Dairy Cows

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Abstract
The underlying molecular pathways must be thoroughly understood to improve milk output in dairy cows. This investigation examines how the blood gene expression patterns of dairy cows with very high and low milk production differ from one another. Ribonucleic acid sequencing (RNA-seq) is performed on blood samples from 35 lactating dairy cows, identifying 50 Differentially Expressed Genes (DEGs) ($p < 0.05$) between the two groups. Functional enrichment analysis reveals that these DEGs primarily involve immune response, inflammatory processes, fatty acid metabolism, and security mechanisms. According to KEGG pathway analysis, the Toll-like receptor (TLR) signalling pathway is the most purposefully created pathway associated with variance in milk output. Using (Quantitative Reverse Transcription Polymerase Chain Reaction) qRT-PCR to validate four sample DEGs, the consistency of the RNA-seq data is validated. Alternative splicing analysis demonstrates distinct splicing patterns between the groups, with alternative 3′ splicing sites predominating in high-yielding cows, while exon skipping is more frequent in low-yielding cows. These findings provide novel transcriptomic insights into gene expression differences linked to milk production and emphasize the contribution of immune-related pathways. The research identifies potential molecular markers that can support non-invasive genetic selection strategies to improve dairy cattle productivity.

Keywords: *Transcriptomics, Differentially Expressed Genes (DEGs), Milk Yield, Ribonucleic Acid* ***sequencing*** *(RNA-seq), Alternative Splicing, Immune Response, Dairy Cattle Breeding.*

1. INTRODUCTION

One of the most economically important characteristics of dairy cattle is milk production, which has a direct impact on the profitability of dairy farms and the supply of dairy products worldwide. Despite advancements in general management techniques, such as better nutrition and housing, milk production can vary significantly, even within the same breed or farm. This difference is not a simple outcome of the external impact of the environment, but it lies deep in the biological world of complicated processes, namely the regulation of gene expression [1]. High-volume, high-quality milk generation is a multifactorial character organized not only by the genetic potential of the cow but also by the coordination of countless genes in a variety of physiological systems. The process in which genetic information present as Deoxyribonucleic Acid (DNA) is transcribed to RNA and translated into proteins, resulting in so-called gene expression, is fundamental to nearly all cellular and system processes, including functions necessary to milk synthesis [2]. Although genetically all cows belong to the same species, the difference in how specific genes are expressed in key organs determines whether a cow produces high-quality milk or low-quality milk. Cows that have a high milk-producing capacity tend to increase the number of genes concerning the levels of the biosynthesis of nutrition, nutrient conveyance, hormonal titration, and immune adjustment, which all promote viable lactation [3]. Gene expression of livestock has been transformed by the utilization of molecular tools in the form of RNA-seq, microarrays, and quantitative PCR (qPCR). The high-throughput methods enable the researcher to compare thousands of genes simultaneously and find out those genes that are DEGs among high and low-milk-yielding cows. Several investigations have discovered that the key genes involved in the synthesis of milk proteins, the metabolism of fatty acids, and the development of mammary glands are overexpressed in high-producing cows [4, 5]. Low-producing cows, on the contrary, are likely to express high levels of genes associated with stress response or even inflammatory processes that could worsen the efficiency of milk production. The mammary gland is the major producer of milk and thus it is the major object of the

gene expression. But to maintain the proper vascular system, supporter tissues like the liver, which controls glucose metabolism and lipid metabolism, and adipose tissue, which controls energy storage and endocrine information exchange, are essential [6]. Synchronized expression of genes in all these tissues provides the cow with enough energy and nutrient requirements required to sustain lactation. Genetic restrictions, metabolic imbalances, or the infirmity arising out of the impediments in this coordination can cause a decrease in milk production as well as health degradation. Notably, gene expression is not determined purely by the gene sequence. It is vulnerable to a variety of epigenetic processes, all of which might be triggered by environmental factors. Nutrition, housing conditions, heat stress, exposure to pathogens, and the lactation stage are the factors that can strongly alter the pattern of gene expression [7]. In this case, when cows are subjected to thermal stress, the production of milk proteins associated with genes is commonly inhibited and there is an up-regulation of disaster proteins. These epigenetic modifications might be temporary or permanent, and sometimes might even be transferred into the offspring, thus modifying the productivity of a herd throughout generations. In practical terms, the knowledge of the differences in gene expression enables animal breeders and selectors with awesome tools. The combination of the data about the gene expression with quantitative genetics allows more precise strategies of genomic selection. Breeding cows can be done not only by monitoring milk production but also by their molecular signature that regulates the expression of major genes that include substantial milk production and strong health [8-11]. This genomic-based selection promotes genetic gains more rapidly, shortens the generation lifecycle, and enhances the general effectiveness and prospects of dairy farms. Besides, the knowledge of genes (including pathways) related to metabolic efficiency, immune response, and fertility along with milk production can enable breeders to prevent trade-offs that are typical of single-trait selection [12]. An example is that highly selecting milk yield without paying attention to gene expression about immune response would predispose the animal to mastitis or abnormality in reproduction. A compromise method of selection based on gene expression profiling can result in the creation of cows that would be not only high producers but also resilient and long-lived. Although this field is marked with considerable achievements, there are numerous questions. The experiments on gene expression in dairy cows have been carried out in different conditions, and in different breeds with unreliable methodologies [13]. Although the various types present valuable information, it is hard to draw uniformity in the results or develop an overarching expression indicator. Additionally, much of the current knowledge is derived from cross-sectional studies. Because dairy cattle's gene regulation is dynamic, longitudinal investigations are necessary to track changes in gene expression throughout the course of the lactation cycle or between parities [14 & 15]. Through a methodical examination of pertinent organs and biological processes, the research investigates the variations in gene expression between dairy cows who produce large amounts of milk and those that do not. By reviewing recent literature and evaluating functional gene networks involved in milk biosynthesis and energy metabolism, this research seeks to provide an integrative understanding of the molecular basis of lactation variability.

Globally, dairy cows are producing more milk, but limiting variables such as feed supplies, rumen and digestive system, tissue mobilization, lactating mammary gland nutrition intake, and the availability of glucose and amino acids are becoming increasingly significant [16]. These limitations could be exacerbated by early lactation, when the body is using high levels of its reserves to start high-volume milk production, combined with low feed consumption. Through the awareness of such limitations, the problem of milk production can become more sustainable and animals can be healthier.

Research looked at how fatty acid supplement mixes with 60% palmitic acid and 30% stearic acid or 30% oleic acid affected the dairy cows' ability to absorb nutrients and produce milk [17]. Based on their milk output, the cows were split into two groups and given treatment sequences at random. The soyhulls from the control group were swapped out for the Fatty Acid (FA) mixes, which were given at 1.5% dry matter. The results showed that total fatty acid reduced dry matter and total-tract FA digestibility, whereas palmitic acid + oleic acid (PA+OA) increased whole-tract FA digestibility. In low-producing cows, FAT increased the digestibility of Dry Matter (DM), whereas PA+SA (palmitic acid + stearic acid) had the same effect.

Plantains are a forage component in dairy grazing systems that can increase milk output and decrease nitrogen losses, according to a meta-analysis [18]. The research discovered that despite preserving the percentage of milk protein and fat, plantain-containing grazing pastures considerably raised milk output, solids yield, and protein yield. It also raised the volume of urine produced each day by 17%, lowered the content of Unsaturated (UN) by 30%, and decreased the total nitrogen output by 22%. Plantain integration into grazing pastures can increase farm output and lessen the environmental effect of dairy farms, according to the investigation.

The research [19] examined the impact of prilled lipid supplements on the nutritional metabolism and milk production of nursing dairy cows. Results showed that Heat Production treatment reduced dry matter intake by 1.9 and 1.1 kg/d, respectively, compared to SA and Control. Interaction increased energy-corrected milk by 2.7 kg/d. The research found that fat supplement composition affects fat absorption, DMI, milk, and milk fat synthesis.

The purpose of the research was to ascertain if giving dairy cows a high-concentrate diet of neutral detergent fiber (peNDF) might lower their chance of developing subacute urinal acidosis (SARA) [20]. Three different food treatments, such as high, medium, or low doses of peNDF8.0, were randomly allocated to thirty second-parity Holstein cows. The research [21] found that increased peNDF8.0 consumption was linked to higher rumen fluid pH, acetate and isobutyrate molar percentages, acetate-to-propionate ratio, and ammonia nitrogen concentration, but no change was observed in volatile fatty acid (VFA) content or butyrate and isovalerate molar percentages.

The research [22] aimed to evaluate the effects of circular feeds on dairy cow meals. Eight mid-lactating cows were divided into four treatments, and the two feedstuffs assessed were wheat distiller's grain with solubles (WDGS) and bakery's former foodstuff (FF). Results showed that the combination of FF and WDGS increased milk production and reduced milk urea level but did not affect fiber digestibility, acetic and propionic generation, or milk composition. Heat stress significantly affected the lactation curve and milk production, indicating the need for alternative feed sources.

The research [23] examined the long-term effects of 3-nitrooxypropanol (3-NOP) on milk production and CH4 emissions in dairy cows. The research involved 64 late lactating Holstein-Friesian cows with varying feed compositions and forage-to-concentrate ratios. The cows were given either a 3-NOP meal or a placebo. The supplementation increased energy-corrected milk and fat-and-protein-corrected milk (FPCM) by 6.5%. 3-Diet type, content, and nutritional value all had an impact on NOP's capacity to lower CH4. Over time, 3-NOP's effectiveness decreased, although erratically.

Gyr and Girolando dairy cows' physiological and behavioral reactions were assessed in the research under both shaded and non-shaded situations [24]. The experiment was carried out at Embrapa Cerrados in Brasilia, Distrito Federal Brazil. According to the findings, cows in shady areas had rumination durations that were 34% longer than those in full light, and they were 1.7 times longer. However, time spent lying in the sun increased by 23%. Regardless of the setting, Girolando cows' panting score was 35% greater than Gyr cows.

Blood samples from dairy calves were used in research that used entire genetic bisulfite sequencing and RNA analysis to find genes that were differently expressed and methylated [25]. According to the findings, 3,601 and 2,802 DEGs corresponded to 10,877 and 6,617 differentially methylated areas, respectively. The research identified DOCK1, PTK2, and PIK3R1 as possible candidate genes for milk production features, which can aid in an improved kind of understanding of how epigenetic alteration affects the milk yield of dairy cows.

Dairy cows are useful sentinel animals for assessing inflammation, metabolic output, and environmental pollutants. The research [26] using summertime O3 and PM2.5 concentrations from Unsaturated Stearic Eicosapentaenoic Acid (US EPA) air quality sensors found that a 10-unit rise in temperature-humidity index (THI) was linked to a 48% reduction in impact. This suggests that dairy cows can be a valuable tool for monitoring health indicators.

The research [27] examined the effect of dietary phosphorus concentrations on feed intake, plasma calcium concentrations, and lactational performance in dairy calves. The research involved 60 Holstein Friesian dairy

cows that were fed diets with varying levels of phosphorus during the dry season and 3.8 or 2.9 grams of phosphorus during the first eight weeks of lactation. The research found that while low-P diets increased the plasma calcium levels of dry cows, they also had the lowest proportion of cows with hypocalcemia. According to the research's findings, low-P diets do not affect DM intake or milk production and might help periparturient cows avoid hypocalcemia.

The research looked at how various plasma total cholesterol (TC) concentrations affected Holstein dairy cows' short-term health during an acute energy deficit [28]. The average TC content of the cows was used to separate them into high and low TC groups. H-Chol cows had higher TC concentrations, whereas low TC cows had lower concentrations. In both groups, there was an increase in plasma NEFA, TAG, and VLDL as well as milk yield, glucose, and insulin. H-Chol cows continued to produce less milk for two more days even after the concentrate was reintroduced. The connection between TC concentrations and fatty acid turnover and adipose tissue mobilization needs more investigation.

Research [29] conducted between September 2020 and April 2021 revealed that the main constraints to milk production include calving season, illness and parasite issues, poor housing conditions, and insufficient fodder area. Additional issues include foot and mouth illness, internal parasites, lack of grazing acreage, summer calving season, cow herd composition, straw shed, and open yard housing. According to the research, increasing animal output and enhancing community livelihood depend heavily on government policy support, subsidies, and sincere dairy-producer cooperation.

The research [30] evaluates the performance of dairy cows fed diets based on traditional or reduced-lignin alfalfa cultivars and examines the effect of energy levels at low MP supply. Results show that Alfalfa cultivars have no discernible effect on any of the examined attributes. Cows fed DMP_DE had lower dry matter intake than cows fed DMP_AE and DMP_EE, but their intakes were not significantly changed. Feeding DMP_EE generated an energy supply similar to AMP_AE, potentially reducing environmental impact.

Research Organization: The research is classified into five sections. In section 1, the historical backgrounds and the importance of milk production traits in milk producing farmers are given. Section 2 provides information about the materials and the methods deployed to conduct the research. Section 3 lists findings including significant upregulated genes, enriched GO biological processes, KEGG pathways and splicing occurrences between high and low-yielding dairy cows. Section 4 adequately discusses the interpretation to the biological relevance of these findings with respect to the lactation performance. Lastly, the research paper ends in the Section 5 that contains a summary of the principal contributions of the research and proposed future research directions to advance genomic selection in the future and dairy productivity.

2. METHODOLOGY

The methodological step was integrated and progressive, starting from careful animal selection and precise sample collection, through robust RNA-seq and rigorous bioinformatics, to targeted functional analyses and experimental validation. Together, these steps provided a comprehensive approach to uncovering the molecular mechanisms linked to milk production in dairy cows. Figure 1 represents the visual depiction of the process involved in the research.

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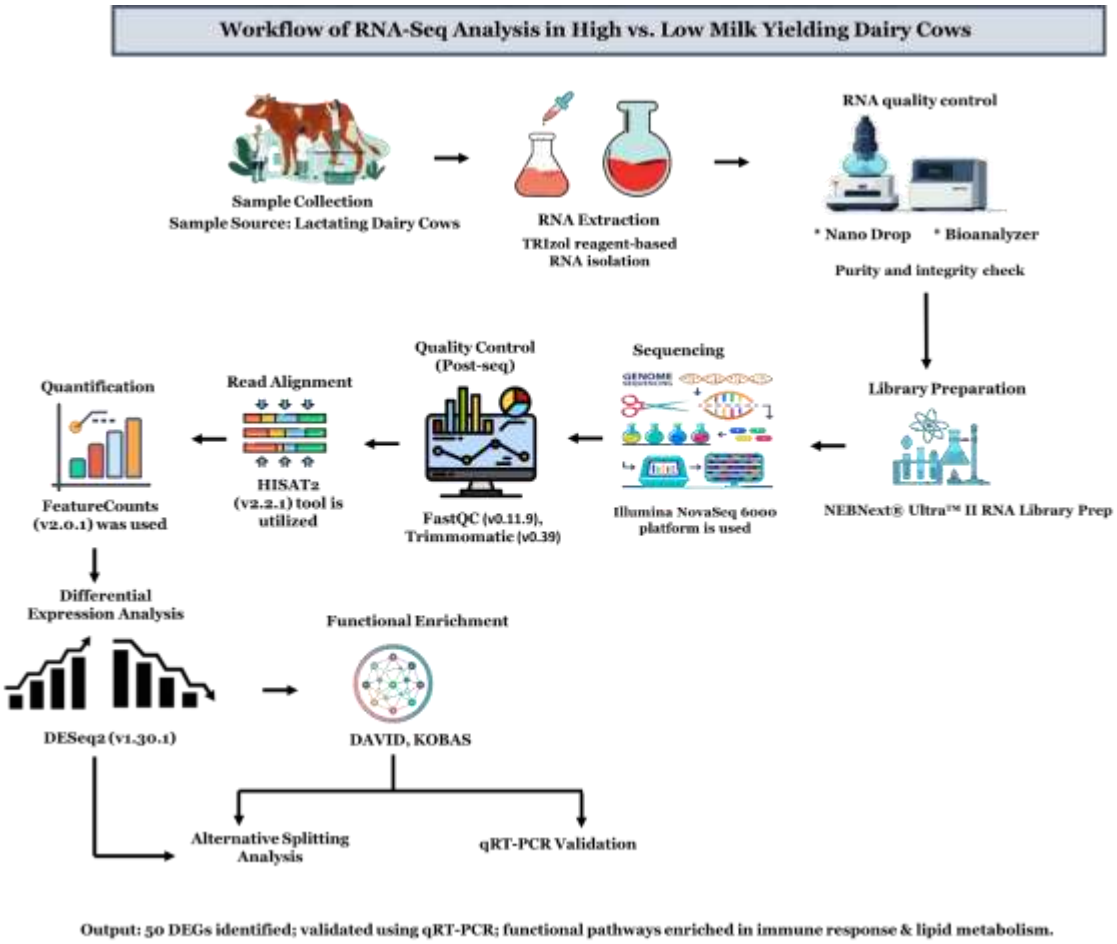

Figure 1: Methodological Framework for Gene Expression and Pathway Enrichment Analysis

2.1 Animal Selection and Sample Collection

The research began by selecting 35 lactating dairy cows from a single herd to minimize environmental variability. These cows were classified into two distinct groups, such as high-yielding and low-yielding, based on detailed milk production records. This classification provides a biological highlight against a downstream molecular analysis. Blood samples were measured with precaution using sterile conditions to avoid contamination and maintain the integrity of RNA to allow sequencing. These samples were used as the source to perform transcriptomic profiles.

2.2 RNA Extraction and RNA Sequencing

Blood sampling was performed and the samples were processed immediately for RNA extraction, where the protocol used to extract total RNA was optimized in the way of blood to ensure high yield and purity. The RNA's quality was examined because only high-quality RNA provides good sequencing data. Sequencing libraries were prepared, after quality confirmation. A high throughput technology was used for the RNA-seq, which produced transcriptome-wide data that enabled extensive connection between the gene expression patterns of the high-yielding and low-yielding cows. The sequencing produced the raw data needed for analyzing gene expression in a new way.

2.3 Identification of DEG

Based on the RNA-seq data, a bioinformatic pipeline has been used to trace the readings with the reference bovine genome and also measure how many readings are mapped to each gene. A statistical measure of difference between the two groups established 50 genes as being differentially expressed significantly ($p <$

0.05). This DEG list included major candidates that might be found to manipulate milk production traits and prepared the platform for functional explanation of such molecular variations.

2.4 Analysis of Functional Enrichment

The enrichment analysis to ascertain the biological importance of the DEGs was carried out using Gene Ontology (GO). To shed light on these DEGs' potential roles in milk production, the research identified biological processes, molecular activities, and cellular components linked to themselves. The outcomes identified that large numbers of DEGs were implicated in immune response, inflammatory processes, fatty acid metabolism, and signifying biological topics of milk yield variation. These enhanced functions implied directions of more specific analysis.

2.5 KEGG Pathway Analysis

For a deeper appreciation of the crucial processes that DEGs are engaged in, such as metabolism or signaling pathways, KEGG pathway enrichment was carried out. TLR signaling pathway was considered to be the most significant enrichment, and this shows that immune-related signaling could be one of the reasons leading to different milk production. It was done to link the changes of single genes with larger sets of molecular networks that can affect lactation biology.

2.6 Endorsement by qRT-PCR

To further support the credibility of the RNA-seq results, four DEGs that reflect the enriched functional groups (e.g. immune function, metabolism) were chosen to be verified by qRT-PCR verification. This method of independence assured that the variances in the appearance of the gene, which were detected using the RNA-seq, could be confirmed, and would be constantly and consistently observed. The validation step therefore boosted the confidence in the transcriptomic findings and the resulting biological interpretations.

2.7 Alternative Splicing Analysis

Finally, the research explored alternative splicing patterns, recognizing that gene expression differences alone might not fully explain yield variation. By analyzing splicing events, the research identified distinct patterns: high-yielding cows exhibited a predominance of alternative 3′ splicing sites, such as events more common in low-yielding cows, events with no clear group predominance, and events more common in high-yielding cows. This additional layer of analysis provided deeper insight into the complexity of transcript regulation and its potential contribution to milk yield traits.

3 RESULTS

Blood sample RNA sequencing from high and low-yielding dairy cows identifies 50 DEGs. These DEGs are mostly linked to fatty acid metabolism, inflammation, and immunological response, according to functional enrichment and KEGG pathway research. The route that was most substantially enriched was the TLR signaling pathway. Validation by qRT-PCR confirmed the differential expression of key genes such as TLR4, SCD, IL1B, and FABP5. Alternative splicing analysis uncovered distinct transcript isoform patterns, particularly an increase in 3' splice site usage in high-yielding cows.

3.1 RNA Extraction, Sequencing, Data Processing, and Validation Workflow

Blood samples were processed within two hours of collection and kept on ice to retain the integrity of the RNA. The TRIzol™ reagent, a commonly used technique for high-quality RNA extraction from blood specimens, was used to isolate total RNA. The amount and purity of RNA were measured using a NanoDrop spectrophotometer, which maintained absorbance ratios of A260/A280 and A260/A230 at about 2.0. Following integrity screening with an Agilent 2100 Bioanalyzer, samples with an RNA Integrity Number (RIN) of 7.0 or above were deemed suitable for downstream library building. The NEBNext® Ultra™ II RNA Library Prep Kit, which featured poly-A selection to enrich for messenger RNA, was used to build the library. The Illumina NovaSeq 6000 platform was used for high-throughput sequencing, which produced 150 base pair paired-end reads with a minimum yield of 40 million reads per sample, guaranteeing extensive transcriptome coverage.

After sequencing, untreated readings underwent quality control using FastQC (v0.11.9), followed by the adaptor and Trimmomatic (v0.39) for removing low-quality sequences. HISAT2 (v2.2.1) was used to align clean reads to the Bos taurus ARS-UCD1.2 reference genome (Ensembl release 104). FeatureCounts (v2.0.1) was employed to measure expression at the gene level. DESeq2 (v1.30.1) in R was used to perform differential gene expression analysis, and genes were deemed significant if their adjusted p-value False Discovery Rate (FDR) was less than 0.05 and their |log2 fold change| > 1. KOBAS (v3.0) was used for KEGG pathway enrichment, while DAVID (v6.8) and g: Profiler for GO keywords were used for functional enrichment of these DEGs. To explore alternative mRNA isoforms, splicing analysis was performed using rMATS (v4.1.0), identifying significant exon skipping, alternative splice site usage, and other splicing events (FDR < 0.05). The chosen DEGs were then confirmed by qRT-PCR using Primer3-designed gene-specific primers, cDNA synthesized with the SuperScript™ III First-Strand Synthesis System (Invitrogen), and qPCR conducted on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher) with SYBR® Green detection, normalizing expression to GAPDH and computing fold changes using the 2^−ΔΔCt method.

3.2 Identification of Differentially Expressed Genes

After sequencing, primary reads were extensively evaluated for quality using FastQC (v0.11.9) to calculate the amounts of sequence duplication and adapter contamination. Low-quality bases and sequencing adapters were removed using Trimmomatic (v0.39), ensuring high-fidelity data for downstream analyses. A quick and sensitive aligner designed for spliced-read mapping in eukaryotic transcriptomes, HISAT2 (v2.2.1), was used to align the resultant clean reads to the Bos taurus ARS-UCD1.2 reference genome.

FeatureCounts (v2.0.1) was used to quantify aligned reads at the gene level, resulting in a count matrix that shows how many reads were mapped to each gene across all samples. This count data was then normalized and statistically analyzed using DESeq2 (v1.30.1) in R. The negative binomial distribution model is used by DESeq2 to account for biological variability and sequencing depth across samples. Genes that satisfied the criteria for both statistical significance and physiologically significant changes in expression were considered to be differentially expressed (FDR) < 0.05 and an absolute log2 fold change more than 1 (|log2FC| > 1). The analysis identified 50 DEGs between the high- and low-milk-yielding collections. Among these DEGs, several genes were of particular interest due to their known roles in immune function, lipid metabolism, and inflammatory responses. For example, (*TLR4)* was significantly upregulated (log2FC = +2.10, FDR = 0.004), suggesting enhanced innate immune signaling in high-yielding cows. *SCD* (stearoyl-CoA desaturase), a key enzyme in fatty acid metabolism, showed increased expression (log2FC = +1.85, FDR = 0.011), possibly reflecting altered lipid processing linked to milk production. *IL1B* (interleukin-1 beta), a pro-inflammatory cytokine, exhibited a strong upregulation (log2FC = +2.30, FDR = 0.002), indicating potential involvement of inflammatory pathways. *FABP5* (fatty acid-binding protein 5) also showed significant upregulation (log2FC = +1.60, FDR = 0.020), which might relate to fatty acid transport and utilization in lactation physiology.

Table 1: Key Upregulated Genes in High Milk-Yielding Dairy Cows

Gene ID	Gene Symbol	Adjusted p-value (FDR)	log2FC
ENSBTAG00000015559	*TLR4*	0.004	+2.10
ENSBTAG00000016157	*SCD*	0.011	+1.85
ENSBTAG00000003189	*IL1B*	0.002	+2.30
ENSBTAG00000017658	*FABP5*	0.020	+1.60

Table 1 shows these findings highlight specific genes and pathways that might contribute to the molecular mechanisms regulating milk production in dairy cows. The DEGs identified provide candidates for further functional studies or potential molecular markers for selective breeding. The log2 rise or decrease for the top four statistically significantly elevated genes in high-yielding dairy cows is shown in Figure 2.

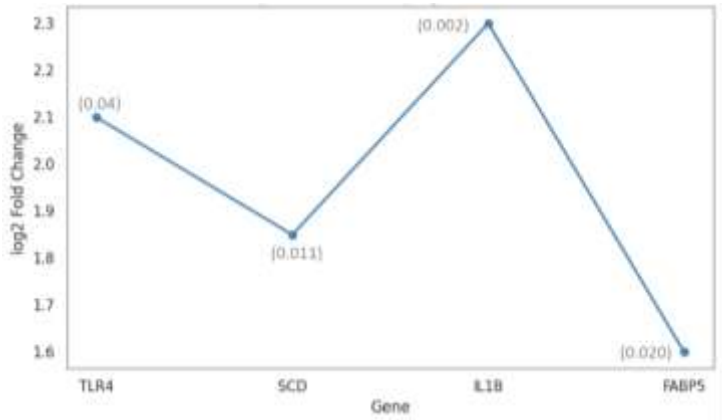

Figure 2: Expression Profile of Top Upregulated Genes

It shows that there is a high induction of immune and lipid metabolism-related genes, in particular, IL1B (log2FC = 2.30) and TLR4 (log2FC = 2.10). The lines indicate a gene and in total, it shows an upregulated gene pattern in biological activities linked to lactation.

3.3 Functional and Pathway Enrichment Analysis

The DEGs' biological importance was clarified by doing enrichment analysis at the GO and KEGG pathway levels. GO enrichment was performed using DAVID (v6.8) and g: Profiler, while KEGG pathway enrichment was analyzed using KOBAS (v3.0). FDR < 0.05 was the enrichment threshold, and statistical significance was assessed in both analyses using the Benjamini-Hochberg method for multiple testing correction.

3.3.1 Gene Ontology (GO) Enrichment

A number of biological processes that were noticeably overrepresented among the DEGs were identified by the GO enrichment analysis. Notably, these included immune response, inflammatory response, fatty acid metabolism, and defense mechanisms. As indicated in Table 2 and illustrated visually in Figure 3, these processes demonstrate the crucial roles that energy metabolism and immunological control play in determining variations in milk output.

Table 2: Enriched Biological Processes (GO Terms) Among Differentially Expressed Genes in Dairy Cows

Description	DEGs in Term	FDR (Benjamini-Hochberg)	GO Term (BP)
Immune response	10	0.003	GO:0006955
Inflammatory response	7	0.006	GO:0006954
FA metabolic process	5	0.012	GO:0006631
Defense response	6	0.008	GO:0006952

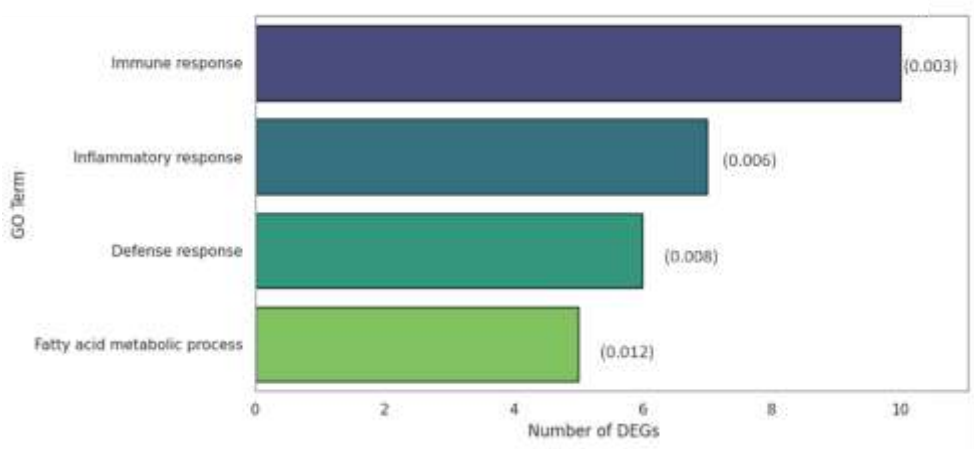

Figure 3: GO Biological Processes Enriched in High Milk-Yielding Dairy Cows

It shows the best enriched biological processes of GO in DEG in high milk-producing cows. The highest enrichment of immune-related pathways was the immune response and the inflammatory response that indicated augmented immune modulation. Besides, the enrichment of the "fatty acid metabolic process" indicates the association between lipid metabolism and the performance of lactation.

3.3.2 KEGG Pathway Enrichment

The pathway enrichment research provided by using KEGG further validation for the functional themes identified in the GO analysis. TLR signalling pathway was shown to be the most significantly enriched pathway, which is consistent with the rise in genes associated with immunity. Other significant pathways included fatty acid metabolism and cytokine-related signaling pathways, highlighting the interconnected roles of immune activity and lipid processing in determining lactation performance, as illustrated in Table 3.

Table 3: Comparing High and Low Milk-Producing Dairy Cows, significantly richer KEGG Pathways Among Differentially Expressed Genes

KEGG Pathway ID	Pathway Name	DEGs in Pathway	FDR (Benjamini-Hochberg)
bta04620	TLR signaling pathway	6	0.003
bta01212	Fatty acid metabolism	4	0.015
bta04060	Cytokine-cytokine receptor interaction	5	0.022
bta04062	Chemokine signaling pathway	4	0.028

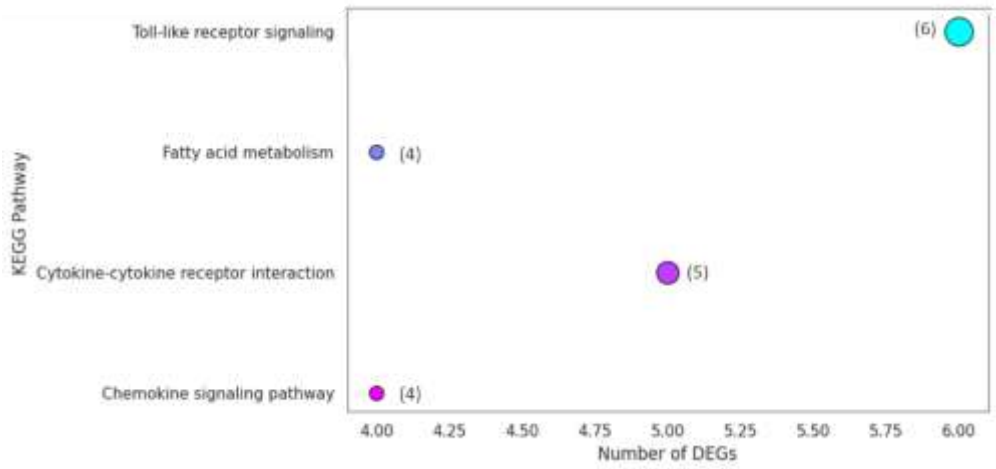

Figure 4: Enriched KEGG Signaling Pathways in High Milk-Yielding Dairy Cows

A highly weighted KEGG pathway with the basis of DEGs in high-milk-yielding cows is represented in Figure 4. The cytokine-cytokine receptor communication and the TLR motioning pathway are significant examples of it, indicating immune signaling overexpression. Also, the enrichment of fatty acid metabolism pathways implies metabolic adaptation that promotes the growth of more milk. Together, these enrichment analyses provide strong evidence that variations in immune signaling and lipid metabolic pathways are central to the changes in yield of milk observed between higher and lower producing cows.

3.4 Validation by qRT-PCR

Four DEGs (TLR4, SCD, IL1B, and FABP5) were chosen for the RNA-seq validation process because of their biological significance and usefulness in the differential expression research. Primers specific to each gene were designed using Primer3 (v0.4.0) and checked for specificity using NCBI Primer-BLAST to ensure amplification of the target sequence without off-target binding. Using the SuperScriptTM III First-Strand Synthesis System (Invitrogen), complementary DNA (cDNA) was created from comparable high-quality RNA

models that were utilized for RNA-seq in compliance with the manufacturer's instructions. qRT-PCR was developed utilizing a Thermo Fisher QuantStudioTM 5 Real-Time PCR System and Applied Biosystems' SYBR® Green Master Mix. To guarantee the accuracy and repeatability of the data, each reaction was carried out three times. The housekeeping gene GAPDH was used as an internal reference to standardize the target genes' expression levels. With the intention to compare the gene expression of the high- and low-yielding cow groups, relative expression was computed using the commonly used 2^−ΔΔCt technique. Table 4 provides a summary of the qRT-PCR primers and corresponding amplicon sizes:

Table 4: Primer Sequences and Amplicon Sizes for Target Genes Used in qPCR Analysis

Genetic factor	Forward Primer	Reverse Primer	Amplicon Size (bp)
TLR4	AGGCTTTGGTGCTGTTCTCT	CCTGAACTCGATGGTTTGGG	120
SCD	CCTGCTGCTTCTCTACCACC	AGGAAGGTGGTCTGGTCTGA	145
IL1B	GCTGAGGAAGATGCTGGTTC	AATTTGCTTGGGATCCACAC	132
FABP5	CTGGTGTTTGCTGGAGGAAG	TCCAGGAAGACGTTGTTGGT	118
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	150

The qRT-PCR validation demonstrated strong concordance with the RNA-seq data, confirming that the selected DEGs were indeed differentially expressed between high- and low-yielding cows. This supports the robustness of the RNA-seq results and the reliability of the identified molecular markers.

3.5 Alternative Splicing Analysis

To further investigate the transcriptomic variation existing between high- and low-milk-yielding cows, rMATS (v4.1.0) was used to analyze the AS events. It is an efficient tool that supports the identification of the changes in AS events using RNA-seq data by considering biological replicates. The analysis concentrated on the existence of five significant groups of alternate splicing:

- Exon skipping (SE): An exon is joined out of the transcript in some isoforms but involved in others.
- Alternative 5′ splice site (A5SS): Variable usage of alternate donor sites at the 5′ end of an exon.
- Alternative 3′ splice site (A3SS): Allocation of different acceptor sites at an exon's 3′ terminus varies.
- Retained intron (RI): In certain isoforms, an intron is preserved in mature mRNA.
- Mutually exclusive exons (MXE): At a specific location, the mature mRNA contains only one of two exons.

Significant splicing events were defined as those with FDR < 0.05. The analysis revealed distinct splicing patterns between high- and low-yielding cows. Notably, alternative 3′ splice site usage was more prevalent in cows with large yields, whereas exon skipping was the dominant splicing event in low-yielding cows. These findings suggest potential regulatory mechanisms at the post-transcriptional level contributing to milk production differences shown in Table 5.

Table 5: Differential Alternative Splicing Events Between High and Low Milk-Producing Dairy Cows

Splicing Type	Number of Significant Events (FDR < 0.05)	Predominant Group
Exon skipping (SE)	35	Low-yielding cows
Alternative 5′ splice site (A5SS)	12	No clear group predominance
Alternative 3′ splice site (A3SS)	18	High-yielding cows
Retained intron (RI)	9	No clear group predominance
Mutually exclusive exons (MXE)	4	Low-yielding cows (minor)

These results indicate that alternative splicing, particularly in exon inclusion/exclusion and splice site usage, might influence the expression of functionally distinct isoforms that contribute to lactation performance. The enrichment of alternative 3′ splice sites in high-yielding cows could reflect fine-tuning of gene function relevant to milk synthesis or secretion.

4. DISCUSSION

Nutrition and the environment are some of the features that contribute to increased production of milk in dairy cows. Research by [22] found that the composition of diets with a high level of fat contributes to better yield and digestibility, and research [24] reported that feed innovation contributes to better yield and digestibility. The importance of the behavior of environmental comfort and metabolic adaptation was discussed by [27]. The approaches, however, do not provide a knowledge of production variability's genetic foundation [28]. In a bid to resolve these limitations, the present research turned to RNA-seq, which interpolated 50 DEGs that are associated with immune response and lipid metabolism. Confirmation of principle regulatory functions by validation using qRT-PCR and pathway enrichment was also established. The molecular solution is an important biomarker in targeted breeding, as well as precision measures to increase dairy outputs.

5. CONCLUSION

Identification of the molecular background of milk production inequality presents significant leads in improving dairy production. A total of 50 DEGs were identified between the highest and lowest yielding cows (adjusted $p < 0.05$, $|\log 2FC| > 1$), with high enrichment in the immune response (FDR = 0.003) and fatty acid metabolism (FDR = 0.012) pathway. With an FDR of 0.003, the KEGG pathway with the highest level of enrichment was the TLR signaling pathway. Quantitative RT-PCR of 4 DEGs (TLR4, IL1B, SCD, and FABP5) demonstrated consistent results with the RNA-seq data in terms of the DEGs correlation. Alternative splicing analysis revealed 78 significant splicing events, which were categorized into three groups: events more common in low-yielding cows, events with no clear group predominance, and events more common in high-yielding cows. While informative, the research is limited by its restrained sample size and dependence on blood RNA. The research can be extended in the future to tissue-specific transcriptomes and multi-omics integration.

REFERENCES

1. Bafandeh, M., Makiabadi, M.J.M., Gharagozlou, F., Vojgani, M., Mobedi, E. and Akbarinejad, V., 2023. Developmental programming of production and reproduction in dairy cows: I. Association of maternal parity with offspring's birth weight, milk yield, reproductive performance, and AMH concentration during the first lactation period. *Theriogenology*, *210*, pp.34-41. https://doi.org/10.1016/j.theriogenology.2023.07.012
2. Mbuthia, J.M., Mayer, M. and Reinsch, N., 2021. Modeling heat stress effects on dairy cattle milk production in a tropical environment using test-day records and random regression models. *Animal*, *15*(8), p.100222. https://doi.org/10.1016/j.animal.2021.100222
3. Ranasinghe, R.M.S.B.K., Deshapriya, R.M.C., Abeygunawardana, D.I., Rahularaj, R. and Dematawewa, C.M.B., 2021. Subclinical mastitis in dairy cows in major milk-producing areas of Sri Lanka: prevalence, associated risk factors, and effects on reproduction. *Journal of dairy science*, *104*(12), pp.12900-12911. https://doi.org/10.3168/jds.2021-20223
4. Wankar, A.K., Rindhe, S.N. and Doijad, N.S., 2021. Heat stress in dairy animals and current milk production trends, economics, and future perspectives: the global scenario. *Tropical Animal Health and Production*, *53*(1), p.70. https://doi.org/10.1007/s11250-020-02541-x
5. Bohlouli, M., Yin, T., Hammami, H., Gengler, N. and König, S., 2021. Climate sensitivity of milk production traits and milk fatty acids in genotyped Holstein dairy cows. *Journal of Dairy Science*, *104*(6), pp.6847-6860. https://doi.org/10.3168/jds.2020-19411
6. Hassanat, F. and Benchaar, C., 2021. Corn silage-based diet supplemented with increasing amounts of linseed oil: Effects on methane production, rumen fermentation, nutrient digestibility, nitrogen utilization, and milk production of dairy cows. *Journal of Dairy Science*, *104*(5), pp.5375-5390. https://doi.org/10.3168/jds.2020-18853
7. Gross, J.J., 2023. Dairy cow physiology and production limits. *Animal Frontiers: The Review Magazine of Animal Agriculture*, *13*(3), p.44. https://doi.org/10.1093/af/vfad014

8. Kim, J.E. and Lee, H.G., 2021. Amino acids supplementation for the milk and milk protein production of dairy cows. *Animals*, *11*(7), p.2118. https://doi.org/10.3390/ani11072118
9. Fontoura, A.B.P., Rico, J.E., Davis, A.N., Myers, W.A., Tate, B.N., Gervais, R. and McFadden, J.W., 2021. Effects of dietary deoiled soy lecithin supplementation on milk production and fatty acid digestibility in Holstein dairy cows. *Journal of Dairy Science*, *104*(2), pp.1823-1837. https://doi.org/10.3168/jds.2020-18797
10. Neave, H.W., Zobel, G., Thoday, H., Saunders, K., Edwards, J.P. and Webster, J., 2022. Toward on-farm measurement of personality traits and their relationships to behavior and productivity of grazing dairy cattle. *Journal of Dairy Science*, *105*(7), pp.6055-6069. https://doi.org/10.3168/jds.2021-21249
11. Melgar, A., Lage, C.F.A., Nedelkov, K., Räisänen, S.E., Stefenoni, H., Fetter, M.E., Chen, X., Oh, J., Duval, S., Kindermann, M. and Walker, N.D., 2021. Enteric methane emission, milk production, and composition of dairy cows fed 3-nitrooxypropanol. *Journal of Dairy Science*, *104*(1), pp.357-366. https://doi.org/10.3168/jds.2020-18908
12. Lim, D.H., Kim, T.I., Park, S.M., Ki, K.S. and Kim, Y., 2021. Evaluation of heat stress responses in Holstein and Jersey cows by analyzing physiological characteristics and milk production in Korea. *Journal of Animal Science and Technology*, *63*(4), p.872. https://doi.org/10.5187/jast.2021.e62
13. Mekuriaw, Y., 2023. Negative energy balance and its implication on productive and reproductive performance of early lactating dairy cows. *Journal of Applied Animal Research*, *51*(1), pp.220-228. https://doi.org/10.1080/09712119.2023.2176859
14. Ramin, M., Fant, P. and Huhtanen, P., 2021. The effects of gradual replacement of barley with oats on enteric methane emissions, rumen fermentation, milk production, and energy utilization in dairy cows. *Journal of Dairy Science*, *104*(5), pp.5617-5630. https://doi.org/10.3168/jds.2020-19644
15. Huang, X., Shi, B., Wang, S., Yin, C. and Fang, L., 2021. Mitigating environmental impacts of milk production via integrated maize silage planting and dairy cow breeding system: a case study in China. *Journal of Cleaner Production*, *309*, p.127343. https://doi.org/10.1016/j.jclepro.2021.127343
16. Gross, J.J., 2022. Limiting factors for milk production in dairy cows: perspectives from physiology and nutrition. *Journal of animal science*, *100*(3), p.skac044. https://doi.org/10.1093/jas/skac044
17. Burch, A.M., Pineda, A. and Lock, A.L., 2021. Effect of palmitic acid-enriched supplements containing stearic or oleic acid on nutrient digestibility and milk production of low-and high-producing dairy cows. *Journal of Dairy Science*, *104*(8), pp.8673-8684. https://doi.org/10.3168/jds.2020-19913
18. Nguyen, T.T., Navarrete, S., Horne, D.J., Donaghy, D.J. and Kemp, P.D., 2022. Forage plantain (Plantago lanceolata L.): Meta-analysis quantifying the decrease in nitrogen excretion, the increase in milk production, and the changes in milk composition of dairy cows grazing pastures containing plantain. *Animal Feed Science and Technology*, *285*, p.115244. https://doi.org/10.1016/j.anifeedsci.2022.115244
19. Shepardson, R.P. and Harvatine, K.J., 2021. Effects of fat supplements containing different levels of palmitic and stearic acid on milk production and fatty acid digestibility in lactating dairy cows. *Journal of dairy science*, *104*(7), pp.7682-7695. https://doi.org/10.3168/jds.2020-19665
20. Cao, Y., Wang, D., Wang, L., Wei, X., Li, X., Cai, C., Lei, X. and Yao, J., 2021. Physically effective neutral detergent fiber improves chewing activity, rumen fermentation, plasma metabolites, and milk production in lactating dairy cows fed a high-concentrate diet. *Journal of Dairy Science*, *104*(5), pp.5631-5642. https://doi.org/10.3168/jds.2020-19012
21. Mammi, L.M.E., Buonaiuto, G., Ghiaccio, F., Cavallini, D., Palmonari, A., Fusaro, I., Massa, V., Giorgino, A. and Formigoni, A., 2022. Combined inclusion of former foodstuff and distiller grains in dairy cows ration: effect on milk production, rumen environment, and fiber digestibility. *Animals*, *12*(24), p.3519. https://doi.org/10.3390/ani12243519
22. M'Hamdi, N., Darej, C., Attia, K., Znaidi, I.E.A., Khattab, R., Djelailia, H., Bouraoui, R., Taboubi, R., Marzouki, L. and Ayadi, M., 2021. Modeling THI effects on milk production and lactation curve parameters of Holstein dairy cows. *Journal of Thermal Biology*, *99*, p.102917. https://doi.org/10.1016/j.jtherbio.2021.102917
23. van Gastelen, S., Burgers, E.E., Dijkstra, J., de Mol, R., Muizelaar, W., Walker, N. and Bannink, A., 2024. Long-term effects of 3-nitrooxypropanol on methane emission and milk production characteristics in Holstein-Friesian dairy cows. *Journal of Dairy Science*, *107*(8), pp.5556-5573. https://doi.org/10.3168/jds.2023-24198
24. Reis, N.S., Ferreira, I.C., Mazocco, L.A., Souza, A.C.B., Pinho, G.A., da Fonseca Neto, Á.M., Malaquias, J.V., Macena, F.A., Muller, A.G., Martins, C.F. and Balbino, L.C., 2021. Shade modifies behavioral and physiological responses of low to medium-production dairy cows at pasture in an integrated crop-livestock-forest system. *Animals*, *11*(8), p.2411. https://doi.org/10.3390/ani11082411
25. Dong, W., Yang, J., Zhang, Y., Liu, S., Ning, C., Ding, X., Wang, W., Zhang, Y., Zhang, Q. and Jiang, L., 2021. Integrative analysis of genome-wide DNA methylation and gene expression profiles reveals important epigenetic genes related to milk production traits in dairy cattle. *Journal of Animal Breeding and Genetics*, *138*(5), pp.562-573. https://doi.org/10.1111/jbg.12530
26. Beaupied, B.L., Martinez, H., Martinez, S., McConnel, C.S., Pollack, I.B., Giardina, D., Fischer, E.V., Jathar, S., Duncan, C.G. and Magzamen, S., 2022. Cows as canaries: The effects of ambient air pollution exposure on milk production and somatic cell count in dairy cows. *Environmental Research*, *207*, p.112197. https://doi.org/10.1016/j.envres.2021.112197

27. Keanthao, P., Goselink, R.M.A., Dijkstra, J., Bannink, A. and Schonewille, J.T., 2021. Effects of dietary phosphorus concentration during the transition period on plasma calcium concentrations, feed intake, and milk production in dairy cows. *Journal of Dairy Science*, *104*(11), pp.11646-11659. https://doi.org/10.3168/jds.2021-20488
28. Gross, J.J., Schwinn, A.C., Müller, E., Münger, A., Dohme-Meier, F. and Bruckmaier, R.M., 2021. Plasma cholesterol levels and short-term adaptations of metabolism and milk production during feed restriction in early lactating dairy cows on pasture. *Journal of Animal Physiology and Animal Nutrition*, *105*(6), pp.1024-1033. https://doi.org/10.1111/jpn.13531
29. Abegaz, S.B., 2022. Milk production status and associated factors among indigenous dairy cows in Raya Kobo district, northeastern Ethiopia. *Veterinary Medicine and Science*, *8*(2), pp.852-863. https://doi.org/10.1002/vms3.740
30. Laroche, J.P., Gervais, R., Lapierre, H., Ouellet, D.R., Tremblay, G.F., Halde, C., Boucher, M.S. and Charbonneau, É., 2022. Milk production and efficiency of utilization of nitrogen, metabolizable protein, and amino acids are affected by protein and energy supplies in dairy cows fed alfalfa-based diets. *Journal of Dairy Science*, *105*(1), pp.329-346. https://doi.org/10.3168/jds.2021-20923