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ESTUDIO DE LA EXPRESIÓN GÉNICA DIFERENCIAL EN VACAS LECHERAS EN EL PERIODO DE TRANSICIÓN CON ALTA Y BAJA ACTIVIDAD DE GLUTATIÓN PEROXIDASA

STUDY OF DIFFERENTIAL GENE EXPRESSION DURING THE TRANSITION PERIOD OF DAIRY COWS WITH HIGH AND LOW GLUTATHIONE PEROXIDASE ACTIVITY

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RESUMEN

Glutatión peroxidasa (GSHpx) es una enzima selenio dependiente que protege a las células del estrés oxidativo, al reducir el H_2O_2 a H_2O. El objetivo de este estudio fue evaluar la asociación entre los niveles de actividad de GSHpx y los perfiles de expresión génica en sangre periférica de vacas lecheras en el periodo de transición. El ensayo se llevó a cabo en un tambo comercial de vacas Holstein, siete días después del parto, se extrajeron muestras de sangre de la vena yugular de animales seleccionados al azar (n = 12). Se determinó la actividad enzimática y los animales fueron separados en dos grupos significativamente diferentes: grupo con menor actividad enzimática (G1) y con mayor actividad de GSHpx (G2). Se realizó un análisis de RNA-seq global en células de sangre periférica, que identificó 1.044 genes diferencialmente expresados (GDE). El enriquecimiento funcional y la ontología genética resultaron en 21 y 26 KEGG *pathways* y GO *terms*, respectivamente. Para evaluar la robustez de estos hallazgos, se incluyó la producción de leche como una covariable y el análisis por diagrama de Venn confirmó que la mayoría de los GDE, GO terms y KEGG pathways iniciales se mantuvieron. El análisis con I-cisTarget mostró 431 *features* enriquecidas (NES > 3), asociados a factores, cofactores de transcripción y cambios epigenéticos. En conclusión, diferencias en la actividad de GSHpx y por ende en los niveles de selenio, se asociaron con un aumento en el número de GDE, KEGG *pathways*, GO *terms*, cambios epigenéticos y regulatorios que están involucrados en cambios en el funcionamiento del sistema y la respuesta inflamatoria. Estos resultados sugieren la existencia de diferentes adaptaciones fisiológicas.

Palabras clave: Estrés oxidativo, GSHpx, Ganado lechero, Holstein, Transcriptoma, Periparto.

ABSTRACT

Glutathione peroxidase (GSHpx) is a selenium-dependent enzyme that helps protect cells from oxidative stress by reduction of H_2O_2 to H_2O. The objective of this study was to evaluate the association between GSHpx activity levels, which is commonly used as an indicator of selenium status, and gene expression profiles in peripheral blood of transition dairy cattle. The study was carried out in a commercial dairy farm of Holstein cattle, seven days after calving, blood samples were obtained from the jugular vein of randomly selected animals (n = 12). GSHpx activity was determined and animals were separated into two significantly different groups according to measured levels, in one group those with lower levels of activity (G1) and in the other those with higher activity (G2). Bulk RNA-seq analysis of peripheral blood cells resulted in 1,044 differentially expressed genes (DEGs). Functional enrichment and ontology analyses revealed 21 and 26 significant KEGG pathways and GO terms, respectively ($p_{adjusted} < 0.01$). To evaluate the robustness of these findings, milk production was included as a covariate and Venn diagram analysis showed that the majority of the initial DEGs, GO terms, and KEGG pathways were consistently shared between both models. I-cisTarget analysis revealed 431 enriched features (NES >3), which were associated with transcription factors, cofactors and epigenetic changes. In conclusion, differences in GSHpx activity levels, which can be associated with differences in selenium levels, lead to an elevated number of DEGs, KEGG pathways, GO terms, epigenetic and regulatory changes, which were associated with the functioning of the immune system and the inflammatory response. These results could be indicative of different physiological adaptations.

Key words: Oxidative stress, GSHpx, Dairy cattle, Holstein, Transcriptome, Peripartum period.

INTRODUCTION

Oxidative stress (OS) is the result of an imbalance between pro-oxidants and antioxidants, and it plays a key role in disease appearance (Forman and Zhang, 2021). High levels of OS can normally be measured in dairy cattle, especially during the *transition period* (Bernabucci *et al.*, 2005), which is the most critical period in the lactating cycle of dairy cows and includes 3 weeks prior to parturition and 3 weeks after (Abuelo *et al.*, 2015). An important antioxidant that is worth mentioning is glutathione peroxidase (GSHpx) which is a selenium-dependent enzyme that helps protect cells from OS by reduction of H_2O_2 to H_2O (Meister, 1988). Additionally, GSHpx can be used in indirect determination of selenium (Se) status (Pavlata *et al.*, 2001), which is very important for health immunity and growth, especially during the transition period (Spears and Weiss, 2008). When Se is adequately available, proper functioning of the immune and reproductive system is maintained, while Se deficiency has negative effects on productive and reproductive parameters of dairy cattle (Mehdi and Dufrasne, 2016), as this mineral functions in the antioxidant system as an essential component of antioxidant enzyme families, which convert H_2O_2 into H_2O, thus reducing OS risk (Mustacich and Powis, 2000). Furthermore, its deficiency has been found to negatively impact activation and proliferation of immune cells, which is related to OS, and other functions such as calcium flux, protein folding and regulation of immune cell signaling (Huang *et al.*, 2012).

Accumulation of reactive oxygen species (ROS) can lead to a disturbance in various signaling pathways, modifying protein production and activity, disrupting crucial biological processes that results in a higher susceptibility to diseases (Forman and Zhang, 2021). In this sense, due to OS, transition dairy cattle stand a greater risk of suffering numerous health disorders such as metritis, mastitis, retained fetal membranes, mammary edemas (Kankofer, 2002; Sordillo and Aitken, 2009). Approximately 75% of diseases that affect dairy herds occur within the first month after calving (Leblanc *et al.*, 2006), the majority being concentrated in the first ten days (Ingvartsen *et al.*, 2003). These diseases result in significant economic losses to the dairy industry, raise animal welfare concerns and are part of the challenges which the international agricultural industries of the 21st century will have to face, as government agricultural policies change (Mulligan and Doherty, 2008).

Transcriptomic technologies can be used to investigate the complex metabolic phenotypes and different adaptive changes in gene expression of multiple molecular networks which occur in the transition period of dairy cattle (Loor, 2010). Several transcriptomics studies have been performed in transition dairy cows including profiling of leukocytes (Batistel *et al.*, 2017), hepatic tissue (Gao *et al.*, 2021; Loor *et al.*, 2005; 2006; 2007), neutrophils (Crookenden *et al.*, 2019), among others. These studies show the complexity of the transition period and how changes in metabolic requirements, as the cow transitions from the dry period to milk production, and the consequent dietary adjustments can produce changes in their gene expression profiles. These modifications can include changes in anti-inflammatory cytokine production, such as transforming growth factor β (TGF-β) and interleukin-10 (IL-10), which can inhibit production of pro-inflammatory cytokines interferon γ (IFN-γ), tumor necrosis factor α (TNF-α) and interleukin-12 (IL-12). Changes in leukocytes and neutrophil function have also been reported. Ultimately, all these factors lead to a high proportion of cows experiencing immunosuppression during the transition period (Aleri *et al.*, 2016).

However, to our knowledge, there is no report associating GSHpx activity, which is commonly used as an indicator of selenium status, and blood gene expression profiles in the transition period in cattle. We hypothesize that dairy cows in the transition period exhibit distinct peripheral blood gene expression profiles that reflect differences in oxidative stress status, and that these profiles are partly associated with varying levels of GSHpx activity, a key antioxidant enzyme. Therefore, the objective of this study was to evaluate the association between GSHpx activity levels and peripheral blood bulk gene expression profiles in transition dairy cows, in order to explore how variation in this antioxidant enzyme activity may influence transcriptional responses during this critical period.

MATERIALS AND METHODS

Animal and experimental design

The study was carried out on a commercial dairy farm of Holstein cows (average annual milk production = 9,600 l) located in General Belgrano, Buenos Aires, Argentina (35°46′00″S 58°30′00″W). The dietary formulation was designed to meet the predicted nutrient requirements according to the Nutrient Requirements of Dairy Cattle recommendations (Supplementary Table 1, Supplementary Table 2; NASEM Dairy Cattle 2021). Further descriptions of body condition and feed given in the different moments before and after calving are detailed in Supplementary information 1. Seven days after calving, blood samples were collected from the jugular vein of randomly selected animals (parity 2 to 5; n = 6 per group; Fig. 1), as this time point falls within the early postpartum period, when cows typically experience heightened inflammatory response, increased oxidative stress, and a transient impairment of immune competence. These physiological changes are well

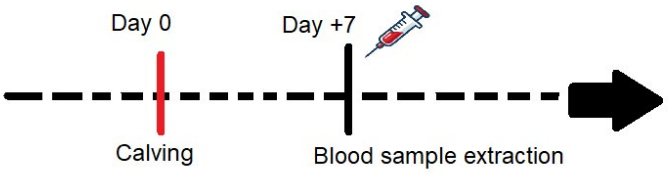

Figure 1. Experimental design. The red vertical bar denotes calving, while the black bar represents blood sampling at seven days post-calving.

documented during the periparturient period (Trevisi and Minuti, 2018). They were collected in Vacutainer® EDTA tubes for GSHpx activity determination, in which erythrocytes were hemolyzed with HPLC water in a 1:4 proportion and then frozen at -70 °C until all the samples were collected (n = 12). When all the samples were collected the tubes were centrifuged separating plasma from the red blood cells, the plasma was extracted leaving the erythrocytes and GSHpx activity was determined by UV-visible spectrophotometry with the Glutathione Peroxidase Assay Kit (Cayman Chemical Company, MI, USA) that reads at 340 nm wavelength and the rate of decrease in $\mathbf{A}_{340}$ is directly proportional to GSHpx activity. Based on this determination animals were separated into two groups according to measured levels of GSHpx activity, in one group those with lower levels of activity (G1) and in the other those with higher activity (G2). Blood samples from each animal were extracted, preserved in RNAlater (Thermo Fisher Scientific, USA) and stored at -80 °C until processing by RNA-sequencing analysis. All animal procedures were performed by a veterinarian and reviewed and approved by the Institutional Committee on Care and Use of Experimental Animals from the School of Veterinary Sciences of the National University of La Plata (Buenos Aires, Argentina; Protocol 123-4-22T).

RNA-seq and differential expression analysis

Blood samples in RNAlater were sent to Novogene Sequencing facility (Sacramento, CA, USA) for RNA extraction with assay and hemoglobin depletion. Bioanalyzer (Agilent, CA, USA) was used to verify RNA quality. After quality control procedures the samples were sequenced in a NovaSeq 6000 sequencing platform (Illumina Inc., CA, USA) and the PE150 strategy. Raw data were filtered to remove low-quality and adapter sequences. Then, the nf-core/RNA-seq project pipeline (Ewels *et al.*, 2020) was used to perform RNA-seq analysis using the ARS-UCD1.2 version as the reference bovine genome. In brief, this workflow includes the following analyses: quality control of reads with FastQC (Andrews *et al.*, 2010), STAR software (Dobin *et al.*, 2013) for alignment to reference genome and FeatureCounts software (Liao *et al.*, 2014) for the process of counting reads. Genes not having in average 10 total reads in G1 and G2 were filtered to delete non-expressed genes and transcripts with low expression levels. Differentially expressed genes (DEG) were identified using the DESeq2 package (Love et al., 2014) in R, with p-values adjusted for multiple testing using the Benjamini-Hochberg procedure to control the false discovery rate (FDR). The primary analysis evaluated the main effect of glutathione peroxidase levels using a baseline model where the $\log_2$-transformed expected expression level ($\log_2 q_{ij}$) was modeled as: $\log_2 q_{ij} = \beta_{io} + \beta_{i1}$ (GSHpx_group$_j$), where β_{io} is the baseline expression for gene i, β_{i1} represents the $\log_2$ fold change associated with the GSHpx_group. To account for the potential confounding effect of milk yield, a secondary analysis was performed by including daily milk production (liters/day) as a covariate in the DESeq2 generalized linear model. This adjusted model was specified as $\log_2 q_{ij} = \beta_{io} + \beta_{i1}$ (GSHpx_group$_j$) + β_{i2} (milk_yield$_j$), where β_{i2} corresponds to the regression coefficient for milk_yield. In this formula, design dictates how the expected gene counts depend on the metadata variables, milk yield controls for the variance associated with individual production, and GSHpx group evaluates the main biological effect of interest while holding the production effect constant. Other phenotypic variables, such as cortisol, antioxidant capacity (AC), thiobarbituric acid reactive (TBARS), blood glucose, and hematocrit levels, were also evaluated as potential covariates. Transcripts were considered to be DEGs with an adjusted p value < 0.01. For gene ontology (GO) analysis, DAVID database (Sherman *et al.*, 2022) was used to obtain enriched GO terms and significant KEGG pathways. Benjamini-Hochberg adjusted p values < 0.01 were considered significant. To evaluate the common DEGs, GO terms, and KEGG pathways between both analyses, Venn diagrams were constructed using ggVennDiagram and ggplot2 R packages. These pathways and GO terms were represented with Cytoscape 3.0 (Shannon *et al.*, 2003). Another analysis was done on the baseline DEGs using the Animal Transcription Factor Database (TFDB) by which Ensembl IDs were separated into three categories: transcription factors (TF), transcription cofactors (TCF) which are necessary components of the transcription complexes (TC) which regulate gene transcription (Wang *et al.*, 2021), and genes which are not involved in the regulation of gene transcription. The results were visualised graphically utilizing Cytoscape 3.0. Finally, DEG Ensembl IDs were converted to their mouse orthologs, a requirement for i-cisTarget, an integrative genomics method which facilitates the identification of enriched regulatory features and regulatory modules (Herrmann *et al.*, 2012; Imrichová *et al.*, 2015).

RESULTS

Phenotypic characterization of the animals.

Two groups of six cows in the transition period were formed based on GSHpx activity. The biochemical determinations showed that one week after calving GSHpx activity was significantly lower in animals from G1 (p value < 0.05; Table 1). Average milk production two weeks after calving was 25.5 l (±9.07) for G1 and 34.2 l (±4.11) G2. No significant differences between groups were observed for cortisol, blood glucose, TBARS, hematocrit, AC, or body condition score (Supplementary Table 3, Supplementary information 1).

Table 1. Biochemical determinations of glutathione peroxidase (GSHpx) activity. Values are expressed as mean ± standard deviation (SD).

	Lower GSHpx activity (G1)	Higher GSHpx activity (G2)	*p value*
GSHpx activity (U/ml PCV)	70.9 (± 10.8)	94.9 (± 15.5)	0.011

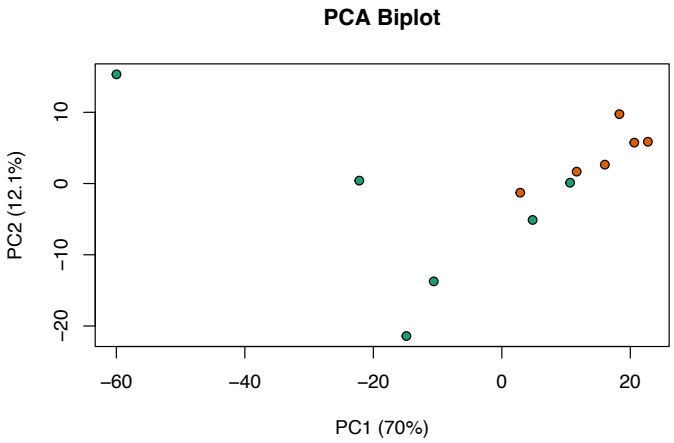

Figure 2. Principal component analysis shows the clustering of samples based on their expression profiles, the green dots are animals with lower glutathione peroxidase activity (G1), and the orange dots are the animals with higher glutathione peroxidase activity (G2). Principal component 1 (PC1) explains the largest proportion of the total variance in gene expression, while principal component 2 (PC2) explains the second largest proportion, capturing additional variability not accounted for by PC1.

Differential expression analysis.

The bioanalyzer measures evidenced that samples from G1 and G2 exhibited an RNA integrity number (RIN) greater than 7.5 (average 8.40). RNA-seq analyses of blood samples resulted in 116.97 million reads. Of these, 96.3% were successfully mapped to the ARS-UCD1.2 bovine reference genome. Low expression transcripts were filtered (< 10 reads in total), with 116,947,762 reads remaining for further analysis.

Based on the expression profiles, Principal Component Analysis (PCA) showed that the two first components accounted for 82.1% of the total variance (PC1 = 70% and PC2 = 12.1%; Fig. 2). PCA discriminated between the two groups (G1 and G2); animals from G2 formed a narrow cloud (positive values for PC1), while G1 was spread out. Heatmap showed similar results, differentiating the two groups with two G1 animals clustered with G2 individuals (Fig. 3). Sample distance matrix evidenced similar grouping patterns (Fig. 4).

RNA-seq reads of the 12 blood samples from G1 and G2 (n = 6 each) detected a total of 14,288 genes after filtering. Differential expression analysis detected 1,044 DEGs ($p_{adjusted}$ < 0.01), 917 were upregulated and 127 downregulated (Fig. 5 and Supplementary Table 4, first sheet). The functional enrichment and ontology analyses revealed 1 downregulated and 25 upregulated significant GO terms ($p_{adjusted}$ < 0.01), and 21 significant KEGG pathways for upregulated genes ($p_{adjusted}$ < 0.01). These pathways and terms included inflammatory response, apoptosis, regulation of GTPase activity, NF-κB signaling pathways, among others (Supplementary Table 5, first sheet). The cytoscape diagram showed the gene interaction network between these KEGG pathways and terms (Fig. 6), being the TNF signaling pathway the only one that exhibits a more complex interaction pattern.

The adjustment of the model to include milk production as a covariate did not attenuate the differential expression signal; instead, it increased to 1,752 DEGs ($p_{adjusted}$ < 0.01), 1,467 upregulated and 285 downregulated genes (Supplementary Table 4, second sheet). The functional enrichment and ontology analyses revealed 96 upregulated and 9 downregulated significant GO terms ($p_{adjusted}$ < 0.01), representing a total of 103 unique GO terms. Additionally, 47 significant KEGG pathways for upregulated genes ($p_{adjusted}$ < 0.01). These pathways and terms also, included inflammatory response, apoptosis, NF-κB signaling pathways and new pathways such as Th17 cell differentiation, ferroptosis, among others (Supplementary Table 5, second sheet). As shown in the Venn diagrams, the majority of the initial DEGs, GO terms, and KEGG pathways were shared between both analyses (Fig. 7 and Fig. 8). This indicates that the inclusion of milk production as a covariate does not significantly alter the initial core findings, confirming the robustness of the baseline model. The other measured phenotypic variables, such as cortisol, AC, TBARS, blood glucose, and hematocrit levels, were not included as covariates because no significant differences were observed between groups (Supplementary Table 3). Moreover, considering the limited sample size

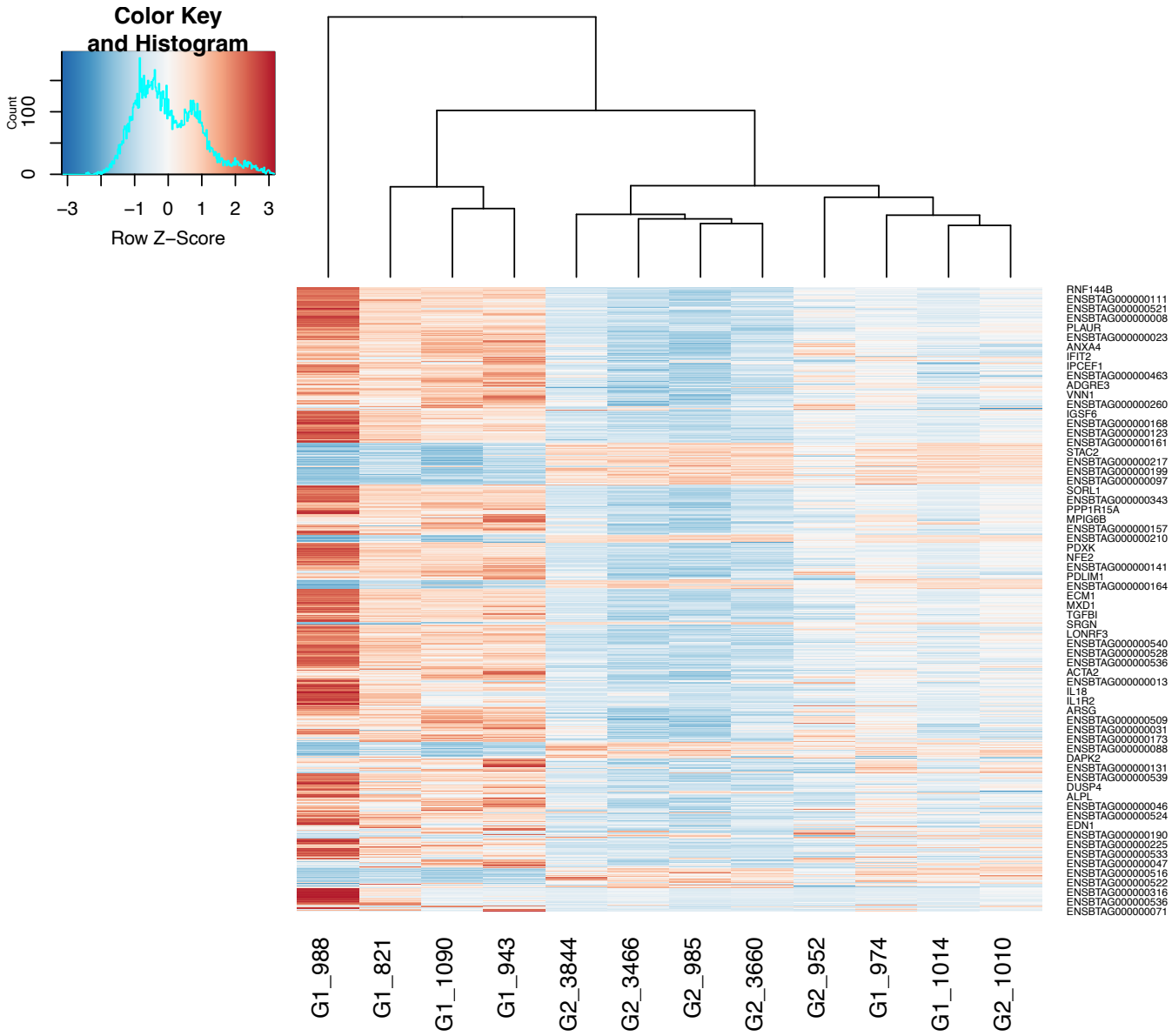

Figure 3. Heatmap which shows gene expression patterns of the different individuals, with red being upregulated genes, blue downregulated. The shade of blue or red indicates the significance of the term or pathway.

commonly associated with transcriptomic studies, the inclusion of multiple covariates may reduce residual degrees of freedom and increase the risk of model overfitting (Babyak, 2004).

Based on the baseline set of 1,044 DEGs, genes were divided into a group comprising TF and TCF and another including genes not involved in transcription regulation, using the animal TFDB (Hu *et al.*, 2019). This analysis resulted in 935 genes (89.56%, 829 upregulated and 106 downregulated), 69 TF (6.6%, 54 upregulated and 15 downregulated), and 40 TCF (3.84%, 34 upregulated and 6 downregulated). TFs included zinc finger and broad-Complex, tramtrack and bric a brac (ZBTB), methyl-CpG binding domain (MBD) gene types, while TCFs were associated with the NF-κB family, lysine demethylase, histone deacetylase, four and a half LIM domain (FHL), among others (Supplementary Table 6). Fig. 9 shows the numerous interactions between TF, TCF and non-regulatory genes.

Finally, DEG Ensembl IDs were converted to their mouse orthologs and processed using i-cisTarget resulting in 431 enriched features (NES > 3.0; Supplementary Table 7). These were related to histone modifications such as H3K4me1/3, H3K27ac activity, CCAAT/enhancer-binding proteins family (CEPBP), TF binding sites such as POL2, PSU and EP300.

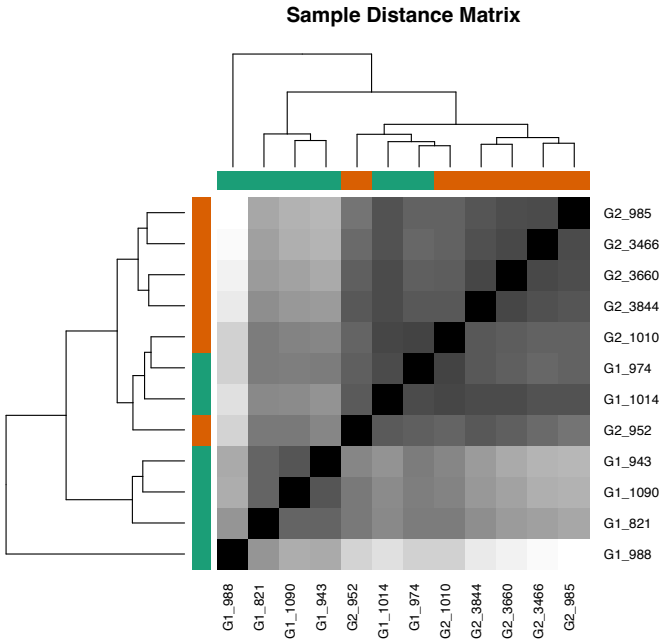

Figure 4. Sample distance matrix shows the clustering of samples based on their expression profiles, the green squares are animals with lower glutathione peroxidase activity (G1), and the orange squares are the animals with higher glutathione peroxidase activity (G2).

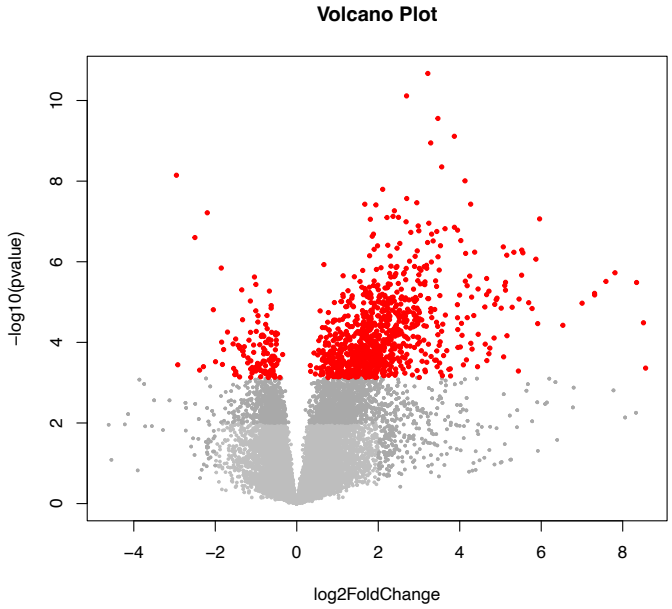

Figure 5. Volcano plot for differential expression analysis between groups of animals with lower glutathione peroxidase activity (G1), and animals with higher glutathione peroxidase activity (G2).

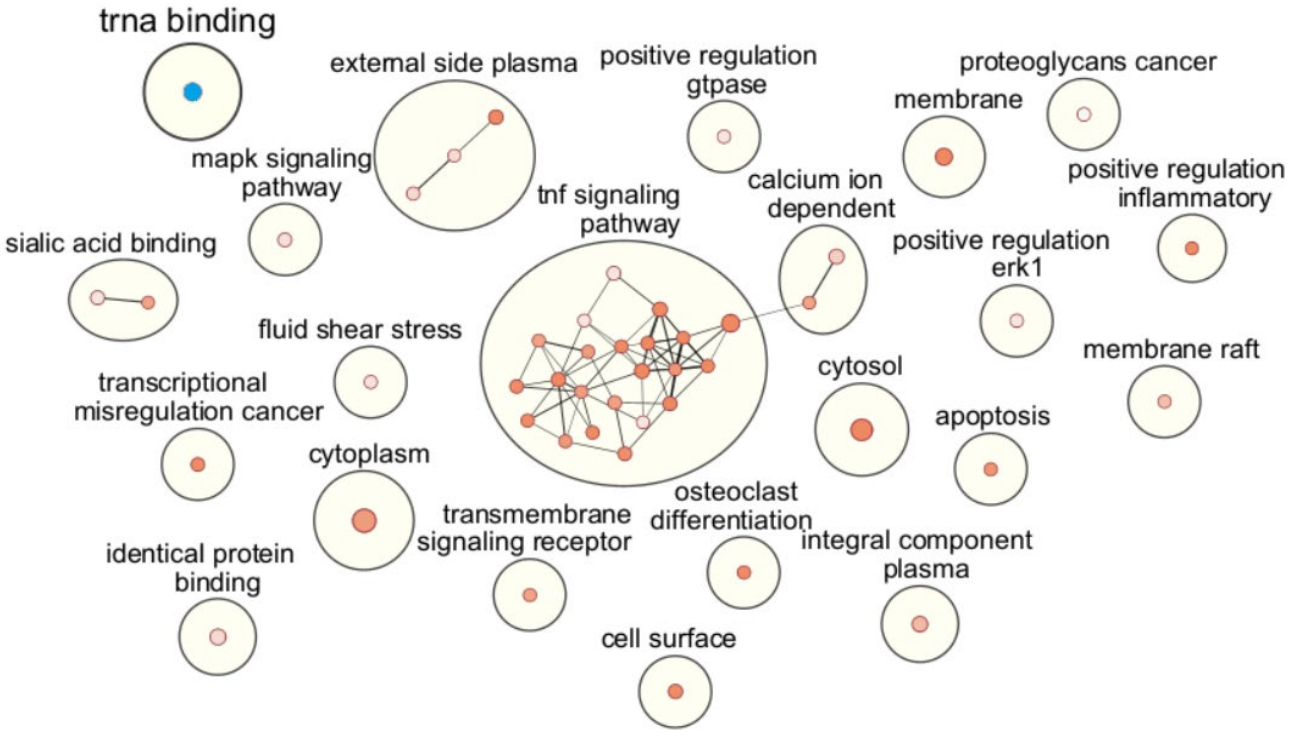

Figure 6. Cytoscape diagram showing the multi-connection between enriched GO terms and KEGG pathways, according to GSHpx activity levels grouping criterion obtained during enrichment analysis when analyzing downregulated and upregulated genes separately. Blue dots and orange dots are downregulated and upregulated pathways and terms respectively. The shade of blue or orange indicates significance of the term or pathway.

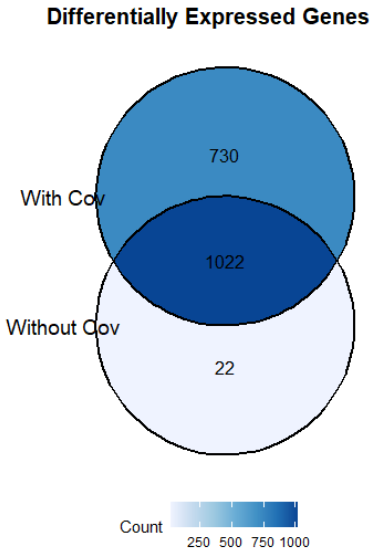

Figure 7. Venn diagram showing the overlap of differentially expressed genes (DEGs) identified by DESeq2 analysis, comparing the baseline model (Without Cov) and the model including milk production as a covariate (With Cov).

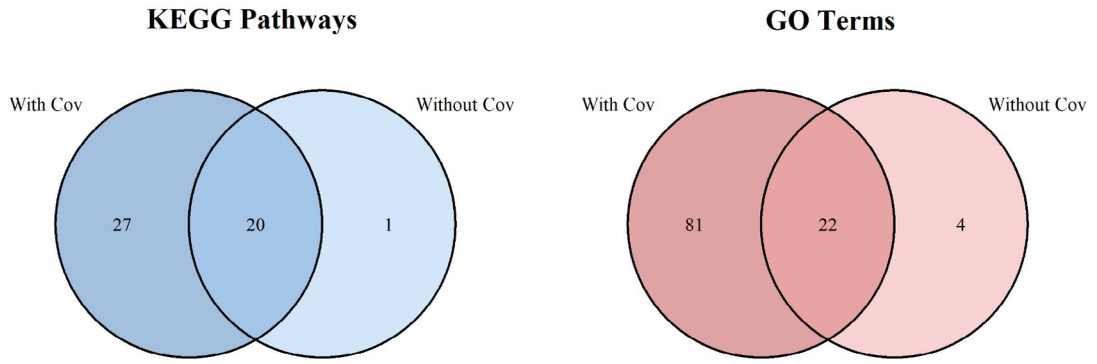

Figure 8. Venn diagram of DAVID functional analysis. The overlap of significant KEGG pathways and Gene Ontology (GO) terms is shown for the analysis without (Without Cov) and with (With Cov) the inclusion of milk production as a covariate.

DISCUSSION

The transition period of dairy cows is characterised by high levels of OS which plays a key role in pathogenesis of diseases and has been identified as an underlying factor of dysfunctional inflammatory responses. Health disorders at this stage cause a major economic impact to dairy farms as they incur in treatment expenses and, more importantly, affected cows will not reach their milk production peak (Abuelo *et al.*, 2015). Therefore, the transition period is a critical moment in the life of a dairy cow as it largely determines health outcomes, production and profitability of the entire herd (Drackley, 1999). Due to its importance, our study aimed to further investigate the metabolic and physiological changes that occur in transition dairy cows by comparing the gene expression profiles in the blood of two groups of animals, G1 and G2, with lower and higher GSHpx activity levels, respectively. This enzyme is an important component of

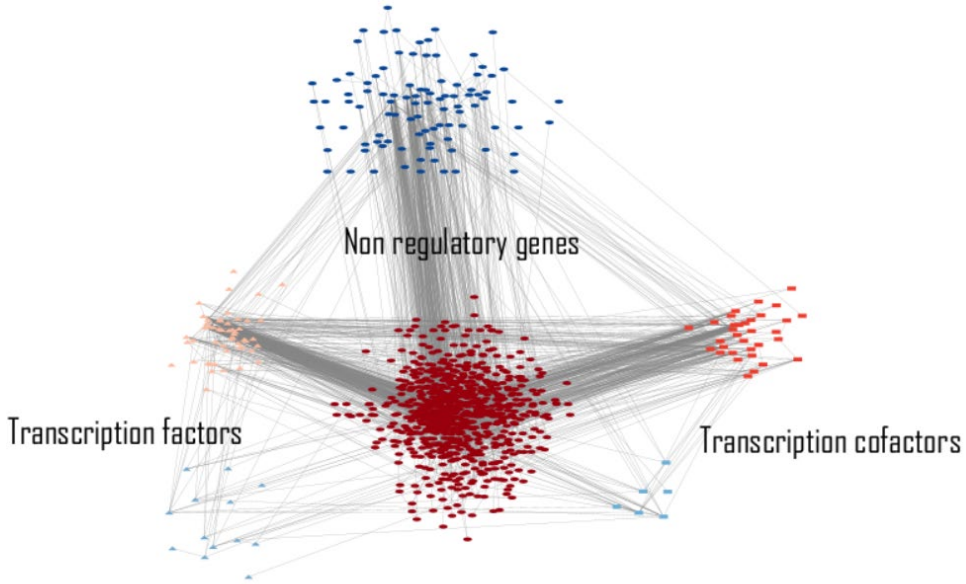

Figure 9. Cytoscape diagram showing the interaction and categories to which the differentially expressed genes belong. Elements with red color are overexpressed, and with blue color are underexpressed. Rectangle and triangle shaped elements are transcription cofactors and factors respectively involved in transcription regulation, oval shaped elements are genes not involved in transcription regulation.

the antioxidant system (Mikulkova *et al.*, 2020), and the measurement of its activity levels is used to indirectly determine selenium status (Ehret *et al.*, 1989).

Principal Component Analysis, heat map and sample distance matrix using RNA-seq data discriminated the two groups of animals, suggesting differences in gene expression levels based on GSHpx activity, thus allowing further analyses. However, the significantly higher milk production in G2 represents a relevant confounding factor. It is well-established that superior milk yield increases metabolic and oxygen demands, naturally leading to enhanced ROS production and the activation of inflammatory pathways such as TNF-α and NF-κB (Abuelo et al., 2015; Respati et al., 2023). Consequently, the 1,044 DEGs, 21 significant KEGG pathways, and 26 GO terms identified in the baseline analysis could reflect a concurrent consequence of the animals' high-performance status and metabolic stress, rather than a direct regulatory effect of GSHpx activity alone. Without considering milk yield as a covariate, the KEGG pathway analysis included terms such as the IL-17 signaling pathway, which plays a key role in infection control, although its unrestrained expression can lead to chronic inflammatory conditions (Monin and Gaffen, 2018). Another significant pathway was NF-κB, which has been widely studied for its role in the immune response to diseases such as mastitis (Khan *et al.*, 2020). This pathway can be activated in response to different stimuli such as ROS and TNF-α, which also evidenced higher expression levels in the group with higher GSHpx activity (Navdeep *et al.*, 2000; Fitzgerald *et al.*, 2007). TNF-α is a potent proinflammatory cytokine with diverse immune and physiological functions participating in processes related to energy metabolism and fever. This signaling pathway was evidenced as the most significant one when the relationship between the different KEGG pathways and GO terms was analysed through Cytoscape (Fig. 6), which showed the importance of this pathway as about half of all significantly enriched terms and pathways were associated with TNF-α. In this sense, Yuan *et al.* (2013) reported that animal administration of this factor leads to a 15 - 18 % decrease of dry matter intake (DMI), water intake, milk fat production, protein content and yield, as well as an increased risk of developing health disorders due to an altered inflammatory response. Additionally, TNF-α mediates antimicrobial and endocrine activity that could alter the metabolism of Fibroblast-like synoviocyte (FLS), which plays a key role in the pathophysiology of joint diseases, such as lameness. These diseases impact animal welfare and have economic implications as they have been associated with different transition period related diseases and reduced feeding time (Kushibiki, 2011; Daros et al., 2020). The most significant GO term detected was inflammatory response, which would make sense as an overt systemic inflammatory response occurs during the transition period in dairy cattle and while essentially all cows experience some degree of inflammation after calving, the magnitude and duration of this response is highly variable among cows and could lead to significant

differences in risk of disease and in milk production (Bradford *et al.*, 2015; Trevisi and Minuti, 2018). Other significant GO terms included neutrophil chemotaxis, cytokine and chemokine mediated signaling pathways, which are related to an innate immune response as neutrophils are recruited due to infection or tissue damage and they respond to different chemo attractants. These molecules are mainly regulated by the Rho family of GTPases, which was found to be differentially expressed in this study (Petri and Sanz, 2018).

Notably, when milk yield was incorporated as a covariate in the differential expression model, the number of detected DEGs and enriched pathways increased, with the number of DEGs increasing to 1,752, and KEGG pathways and GO terms rose to 47 and 103, respectively. Crucially, this adjustment led to an increase in the number of DEGs from 917 upregulated and 127 downregulated genes in the baseline model to 1,467 upregulated and 285 downregulated genes in the adjusted model. Despite this change, 1,022 DEGs of the original 1,044 remained and 730 new ones were added when milk production was included as a covariate. Regarding functional enrichment, of the 21 significant KEGG pathways, 20 remained, with only fluid shear stress being eliminated, while 27 new pathways were added which included pathways such as leukocyte transendothelial migration, Ferroptosis, T cell receptor, autophagy. Similarly, 22 of the original 26 GO terms remained significant. The terms integral components of plasma membrane, chemokine-mediated signaling pathway, cytokine receptor activity, and positive regulation of GTPase activity were excluded, while 81 new GO terms were identified. These new terms comprised processes such as innate and adaptive immune responses, T cell receptor signaling, and negative regulation of inflammatory response, among others. The expansion of DEGs, GO terms, and KEGG pathways, coupled with the high degree of consistency regarding the original functional categories, supports the consistency of the observed biological signals, suggesting that part of the observed transcriptomic variability may be associated with GSHpx-related differences rather than milk production alone.

This study also evidenced a significant number of TF and TCF which conform transcription complexes (TC), suggesting epigenetic and regulatory changes. These TC regulate gene expression through conformational modifications of chromatin and histones in response to cellular signals and they are also involved in post-translational modifications (Wang *et al.*, 2021). Deregulation of these molecules can be the cause for different diseases and metabolic disorders and their activity and expression can modulate various physiological processes, being this delicate interplay essential for maintaining homeostasis. Furthermore, given their characteristics, these molecules could be potential therapeutic targets (Talukdar and Chaterdji, 2023). These differentially expressed TF and TCF interact with each other and with their target genes (Fig. 9). BCL6 was one of the differentially expressed TF, this gene is the master regulator of B cell germinal centers playing a key role in antibody generation (Klein and Dalla-Favera, 2008). Another significant TF was RUNX1 which if overexpressed may lead to mitochondrial dysfunctions and induce cell apoptosis by activating the PI3K/AKT signaling pathway (Liu *et al.*, 2023). Differentially expressed TCF were related to lysine demethylases and acetyltransferases, histone deacetylases and NF-κB pathways. Analysis using i-cisTarget evidenced 431 enriched features (NES> 3.0; Supplementary Table 7) of which the most significant was related to H3K4me3 which is ROS sensitive and important in the stress response, contributing to the variation in lifespan (Bazopoulou *et al.*, 2019). Furthermore, features related to H3K4me1, H3K9me2, H3K4me2, H3k27ac, H3K9ac, H3K36me3 were also enriched; these epigenetic modifications are sensitive to OS and they modulate the transcription of diverse antioxidants such as glutathione and superoxide dismutase. They can also regulate ROS and inflammatory factor production (Yi *et al.*, 2022). Other enriched features that are worth mentioning are the CEBP (A/B/D/E/G) protein family, which amounted to approximately 80 features. CEPBPA participates in the inflammatory response and plays a key role in myeloid differentiation (Zhou *et al.*, 2019), similarly CEBPB/D/E/G function as a TF in many processes, such as immune responses by activating inflammatory factors such as interleukins, TNF-α, metabolism, immunity, cell differentiation, among others (Ko *et al.*, 2015, Zahid *et al.*, 2020, Lekstrom-Himes and Xanthopoulos, 1999).

Previous works studied gene expression in transition dairy cows using different experimental models to compare leukocyte, hepatocyte and endometrial cell expression profiles of animals before and after parturition, under different diets such as ad-libitum or energy restricted diets or comparison of organic/inorganic supplementation. Interestingly, all these works share a differential expression of genes related to immunity, inflammation, stress and metabolism (Loor *et al.*, 2006; Loor *et al.*, 2007; Steele *et al.*, 2015; Osorio *et al.*, 2016; Batistel *et al.*, 2017; Minuti *et al.*, 2020). Although these studies did not share the same experimental design and they are focused on the analysis of different factors and stressors, they shared changes in similar pathways, such as immune and oxidative stress response, among others, in the transition period of dairy cows.

The changes in gene expression evidenced in the different KEGG pathways, GO terms and enriched features based on enzyme activity can be associated with Se status given that measurements of GSHpx activity are used to determine the condition of a herd (Pavlata *et al.*, 2001). The effect of these changes would coincide with

reported Se functions and the consequences its deficiency evokes. This mineral is an essential dietary component as it plays key roles in the antioxidant system of dairy cows, their productive performance, and it has critical functions in the immune, inflammatory and metabolic systems. Adequate Se levels have been associated with increased DMI and higher GSHpx activity levels (Respati *et al.*, 2023). High DMI is especially necessary in transition dairy cows to mitigate, as much as possible, the negative nutrient balance which characterises this period. The severity of this negative balance has direct implications on cow health and production, if it is excessively negative it can result in different disorders and an increased risk of infection (Grummer *et al.*, 2004). On the other hand, GSHpx is an essential antioxidant enzyme, protecting cells against ROS, an imbalance between pro-oxidants and antioxidants increases OS risk which compromises the correct functioning of the immune system and other metabolic processes (Sordillo, 2013; Abuelo *et al.*, 2019). In conclusion, significant differences in GSHpx activity levels between G1 and G2, potentially reflecting differences in Se status, were associated with an elevated number of DEGs, KEGG pathways, GO terms, and epigenetic and regulatory changes. A high proportion of these detected genes and features were associated with the functioning of the immune system and the inflammatory response, both of which are critical points of the transition period (Trevisi and Minuti 2018, Bradford *et al.,* 2015). Although milk production contributed substantially to transcriptomic variability, the high overlap between baseline and adjusted models suggests that the observed biological patterns were not exclusively driven by production differences. The differences in gene expression observed between the studied groups of cows could be indicative of different physiological adaptations which could lead to differences in animal performance, health and welfare. However, certain limitations of the present study should be acknowledged. PCA and hierarchical clustering revealed a noticeable degree of biological dispersion within the lower GSHpx activity group (G1), with a few individuals clustering closely alongside the G2 cohort. This within-group variability likely reflects the highly dynamic and multifactorial nature of the bovine transition period, during which individual metabolic conditions and subclinical health status may transiently influence peripheral blood gene expression profiles. This phenomenon may have been further compounded by the use of a single sampling time point. Therefore, future studies incorporating larger cohorts to better account for individual confounding factors, together with longitudinal designs including multiple sampling time points, are warranted to more comprehensively characterize the temporal dynamics of this transcriptomic signature. Furthermore, the integration of proteomic and metabolomic approaches would help determine whether the observed transcriptomic differences translate into biologically meaningful changes in protein expression and metabolic function.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The transcriptomic data used in this study are available from GEO https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE231491.

ETHICS STANDARDS

All animal procedures were reviewed and approved by the Institutional Committee on Care and Use of Experimental Animals (CICUAL) from the School of Veterinary Sciences of the National University of La Plata (Buenos Aires, Argentina; protocol 123-4-22T).

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Supplementary Tables