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Early life programming of the neonatal bovine jejunum in response to maternal vitamin and mineral supplementation

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Abstract

We investigated the effects of maternal vitamin and mineral supplementation throughout gestation on gene expression in the jejunal mucosa of neonatal calves. Crossbred Angus heifers ($n = 14$) were estrus synchronized, bred to female-sexed semen, and randomly assigned to a basal diet (Control, CON; $n = 7$) or the basal diet plus vitamin and mineral supplement (Treatment, VTM; $n = 7$). After parturition, calves were removed from their dams before suckling, fed colostrum replacer, and euthanized 30 h after the first feeding. A subsample of the mucosa of the mid-jejunum was collected, and total RNA was isolated. Gene expression was measured using RNA-Seq, and differentially expressed genes (DEGs) were identified using DESeq2. We identified 528 DEGs from the jejunal mucosa between the VTM and CON calves ($P \leq 0.05$ and $|\log2FC| \geq 0.5$). The DEGs were associated with nutrient transport, lipid metabolism, and immune-related biological processes and pathways. Interestingly, genes underlying the complement and coagulation cascades were mostly downregulated in calves born to VTM dams. On the other hand, the cytokine-cytokine receptor interaction KEGG pathway showed most genes upregulated (*LIFR, KDR, TNFRSF4, TNFSF18, FLT1,* and *TNFRSF12A*). Our results show that vitamin and mineral supplementation throughout gestation affects genes underlying tissue structure, nutrient transport and metabolism, and immune system pathways in neonates. The implications of such changes and the long-term outcomes on herd health and performance warrant further research.

Introduction

Vitamins and minerals are micronutrients required by the body in small amounts to achieve homeostasis and various physiological processes, such as metabolic regulation, immune function, and modulating gene expression.[1–3] The availability of these nutrients is often limited by forage quality, requiring supplementation by beef producers.[4] However, despite ongoing research into their critical role in fetal development, vitamin and mineral supplementation strategies are largely variable among producers.[5–7] Maternal nutrient requirements vary during gestation due to the increased demand for nutrients to supply the growing fetus.[8] We have shown that supplementation of vitamins and minerals during the periconceptual period to beef heifers through the first 83 days of gestation increased the concentrations of trace minerals in the fetal liver and muscle.[2,6] Similarly, vitamin and mineral supplementation to cow-calf pairs grazing native range pastures increased Se, Cu, and Co liver concentrations at pasture removal and weaning for cows and suckling calves compared with the non-supplemented cohort.[9]

Nutritional management during gestation can positively or negatively impact the offspring's development due to the *in utero* programming of organ systems.[10–12] In ruminants, altered small intestinal mass, villi morphology, hypertrophy, hyperplasia, vascularity, and gene expression were affected due to maternal overnutrition or nutrient restriction.[13] Vitamin and mineral supplementation combined with a moderate rate of body weight gain to heifers during early gestation increased the fetal small intestinal weight compared with fetuses from supplemented heifers managed at a low rate of gain.[6] Furthermore, we have shown that the hepatic expression of energy- and lipid-related genes in these fetuses was differentially programmed.[14] These findings suggest that compensatory mechanisms within the developing fetus may regulate the growth rates of metabolic organs, potentially adapting to adjust energy utilization.[6] Such adaptations may have lasting implications on the offspring's body composition outcomes due to

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compromised metabolic programming.[8,15] Offsprings born to VTM supplemented dams from a similar cohort used in the current study were heavier than CON from weaning through 15 months of age. These adaptations were also observed in greater blood glucose at birth and altered feeding behaviors in VTM offspring compared with CON.[7]

We have shown considerable effects on the programming of fetal tissues, metabolism, and placental development in response to maternal nutrition.[16–19] Changes in visceral organs during pregnancy may have long-lasting effects on the production efficiency of the offspring. Visceral organs, such as the jejunum, begin development by day 26 of gestation[1] and their development and function may also be programmed *in utero*.[13] The jejunum is the main site of absorption of nutrients [20] and has a high metabolic activity and rapid turnover.[21] Furthermore, the intestinal immune system plays a key role in pathogen recognition and immune response.[22] Despite its importance, the role of maternal nutrition in the programming of the offspring jejunum remains largely underexplored.[13]

The observed outcomes of vitamin and mineral supplementation on postnatal growth performance, together with our previous findings under a similar experimental model,[7,23] led us to investigate the effects of maternal vitamin and mineral supplementation on the gene expression profile of jejunum in neonatal calves. We hypothesized that the maternal vitamin and mineral supplementation throughout gestation would differentially program the jejunal mucosa through changes in the expression of genes involved with immune response, nutrient uptake, and metabolism. Herein, through a transcriptomics approach, we identified differentially expressed genes in the jejunal mucosa of newborn calves, as well as the underlying biological processes affected by maternal vitamin and mineral supplementation. Additionally, we measured gene-gene interactions to uncover regulatory genes in response to maternal diet. Our findings show that the maternal vitamin and mineral supplementation throughout gestation affected genes regulating pathways such as the intestinal immune network for IgA production, cytokine-cytokine receptor interaction, fat digestion and absorption, regulation of immune response, and inflammatory response.

Materials and methods

Care and use of animals

All animal procedures were approved by the North Dakota State University (NDSU, Fargo, ND, USA) Institutional Animal Care and Use Committee (#A21047).

Animals, housing, and diet

The development of the experimental model and study design were previously reported.[7,23] In brief, 72 crossbred Angus-based heifers (~ 14 to 15 months of age, initial body weight (BW) = 380.4 ± 50.56 kg; standard deviation) were used to determine the influence of vitamin and mineral supplementation throughout gestation on offspring development. Following a 14 d acclimation to an electronic individual feeding head-gate system, heifers were estrus synchronized using a 7-day Select Synch + CIDR estrus synchronization protocol[24] and bred via artificial insemination (AI) using female-sexed semen from a single sire. After AI, heifers were blocked by BW and randomly assigned to receive either a basal diet (control; CON; $n = 36$) or a basal diet plus a vitamin and mineral supplement (VTM; $n = 36$). Heifers pregnant to the first AI were used in a different experiment.[7] Within their respective treatment groups, heifers not pregnant after the first AI (CON, $n = 19$; VTM, $n = 18$) were resynchronized as described above and bred via AI 60 d after the first insemination, and used for the current study.[23]

Treatments were applied from 60 d pre-breeding through calving. However, the vitamin and mineral supplement provided from pre-breeding to day 239 of gestation was a loose product (Purina Wind and Rain Storm All Season 7.5 Complete, Land O'Lakes, Inc., Arden Hills, MN, USA; Supplementary Table S1) top-dressed over the basal diet. From day 240 of gestation, the vitamin and mineral supplement was added into the total mixed ration (TMR) for the VTM treatment group. Diet compositions were described elsewhere[23] and are reported in Supplementary Table S1. Individual feed deliveries were monitored through a Calan head-gate system and adjusted during gestation to achieve targeted BW gains of 0.45 kg•heifer-1•d-1 through day 200. After that, feed deliveries were altered to allow for *ad libitum* daily intake in preparation for calving.[23] Individual intake from day 240 was monitored via the Insentec feeding system (Hokofarm Group B.V., the Netherlands). At parturition, calves were promptly removed from their dams before suckling and were housed in individual pens. Within 2 h of birth, calves were fed 1.4 L of commercial colostrum replacer (LifeLine Rescue High Level Colostrum Replacer, APC, Ankeny, IA, United States) via an esophageal feeder. At 12 and 24 h after the initial colostrum feeding, calves were fed 2 L of milk replacer (Duralife 20/20 Optimal Non-Medicated Milk Replacer, Fort Worth, TX) using an esophageal feeder. At 30 h after the initial colostrum feeding, 14 heifer calves ($n = 7$ per group) were euthanized via captive bolt and exsanguination. Organs were collected, weighed, and a subsample of mid-jejunum was harvested. The sampling location followed the methods described by Trotta et al.[25] In brief, the small intestine was removed at the pyloric and ileocecal junction, representing portions of the duodenum and ileum, respectively. The remainder of the small intestine was measured and cut in half to represent the proximal and distal jejunum. One-meter segments were cut and sampled from the midpoint of the previously cut sections.[25] The jejunal mucosa was scraped using a glass microscope slide, snap-frozen on dry ice, and stored at − 80°C.

RNA extraction, library preparation, sequencing, and data processing

Total RNA was extracted from the jejunal mucosa ($n = 7$, VTM; $n = 7$, CON) using the RNeasy kit (Qiagen Germantown, MA, USA), followed by on-column DNase treatment, according to the manufacturer's protocol. The integrity and quality (IQ) of the RNA samples were assessed with the Qubit RNA IQ Assay kit (ThermoFisher Scientific), agarose gel electrophoresis, and the Agilent 2100 Bioanalyzer. All samples met the required quantity and quality parameters for library preparation (IQ ≥ 8). Strand-specific RNA libraries were prepared using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (New England BioLabs®, Ipswich, MA, USA) with PolyA mRNA enrichment. Paired-end libraries were sequenced on the Illumina® NovaSeq 6000 platform, with 150 bp reads, and a 20 M reads/sample depth. Novogene Co., Ltd (Nanjing, China) performed the library preparation and sequencing.

Raw data quality control was based on filtering out sequencing adaptors and reads with a Phred-Score lower than 30. FastQC v.

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0.11.9 [26] and MultiQC v. 1.10.1[27] were used to perform data quality control and read statistics of the raw reads. The reads were mapped to the ARS-UCD1.2. (release109) *Bos taurus* reference genome using the STAR aligner v.2.7.5.[28] We counted the mapped reads using the *quantMode GeneCounts* flag from STAR.[28] Post-mapping quality control was conducted with MultiQC and edgeR v. 4.0.3.[29]

Differential expression, regulatory transcription factors, and co-expression network analyses

Genes with zero or low-count expression were filtered out using the *filterByExpr* function from edgeR.[29] A principal component analysis was performed using the factoextra v1.0.7 [30] R-package to identify potential batch effects. To identify differentially expressed genes (DEGs), we used DESeq2 v. 1.42.0,[31] which applies a negative binomial distribution to model the RNA-Seq data. A pairwise comparison was performed between the VTM and CON groups. Genes with a $P \leq 0.05$ and absolute log2 fold change ≥ 0.5 were considered significant. The DEGs were classified as up or downregulated based on the sign of the log2 FC in the VTM group. Gene annotation was performed using BiomaRt v. 2.58.2[32] based on the bovine ARS-UCD1.2. (release109) reference from the Ensembl database. To visualize the DEGs, a volcano plot was constructed using EnhancedVolcano plot v. 1.20.0 R-package.[33]

To identify transcription factors (TFs) modulating the differential expression between VTM and CON groups, we used the regulatory impact factor (RIF) algorithms, RIF1 and RIF2.[34] RIF1 assigns a high score to TFs that are most differentially co-expressed with the highly abundant and differentially expressed genes, while RIF2 assigns a high score to TFs whose expression can predict better the abundance of DEGs.[34,35] To this end, all genes tested for differential expression were normalized using the *VST* function from DESeq2. Then, after mining the AnimalTFDB bovine database v.4.0,[36] we filtered out those TFs that were not expressed in our dataset (n = 367). The remaining TFs (n = 1,078) were contrasted with the DEG list. The analysis was implemented in FORTRAN 90 based on the source code available from the RIF's main author,[34] as previously described.[16,34] Key TFs were selected considering either one of the two RIF scores was greater than |1.96| of the standard deviation ($P \leq 0.05$).[16,34] The co-expression profile of gene pairs for DEGs and TFs was created based on the partial correlation and information theory (PCIT) algorithm.[37] Significantly co-expressed pairs were selected based on a partial correlation value greater than |0.8| ($P \leq 0.05$). Cytoscape v.3.9.0 was used for network visualization.[38]

Functional over-representation and gene set enrichment analyses

To understand the biological roles of the genes in response to the maternal diet, we used two approaches for functional analysis. First, based on the DEGs identified between VTM and CON groups, we performed an over-representation analysis using ClueGO 2.5.10.[39] Redundant terms were grouped based on the kappa score = 0.4.[39] The *P*-value was calculated and corrected with a Bonferroni step-down ($P \leq 0.05$). Over-represented KEGG pathways and Gene Ontology (biological processes) terms were identified and considered significant when $P \leq 0.05$. These analyses were carried out based on the *B. taurus* annotation, and the network visualization was performed on Cytoscape version 3.9.0.[38]

Second, we used a functional class scoring approach based on the Gene Set Enrichment Analysis (GSEA) method considering on all genes tested for differential expression. This approach was used to identify group of genes (gene sets) acting on common pathways rather than DEGs individually.[40] The following equation was used to rank the genes: $rank = [sign\ (log_2FC) \times -log_{10}(p-value)]$.[41] Considering this equation, the sign of the ranking (positive or negative) will be given by the fold-change, whereas the magnitude will be given by the *P*-value.[41] Thus, instead of analyzing individual genes, GSEA evaluates whether predefined gene sets from specific pathways or processes are over-represented among the top or bottom of our ranked gene list.[40,42]

The GSEA analysis was implemented through the WebGestalt (WEB-based Gene SeT AnaLysis Toolkit)[43] to identify over-represented KEGG pathways at the top or bottom of the ranked list of genes.[40] Based on the GSEA assumptions, the degree of enrichment is represented by the normalized enrichment score (NES). The NES is the enrichment score for the gene set after it has been normalized across analyzed gene sets. A significant positive NES value indicates that members of the gene set tend to appear at the top of the gene list (positive fold change), while a significant negative NES indicates the opposite.[40]

Results

VTM supplementation throughout gestation affects maternal and neonatal mineral reserves

The effects of the maternal diet on the dam and neonatal trace mineral concentrations and body weight (BW), blood metabolites, and neonatal organ mass were previously reported.[23] These results are outlined below to give the reader a background and contextualize the current findings. By design, BW for dams was similar ($P \geq 0.25$) throughout gestation (CON = 510.1 ± 57.99 kg, VTM = 528.0 ± 65.86 kg). Likewise, VTM supplementation did not affect maternal BW at calving (CON: 558 ± 49.1 kg; VTM: 534 ± 34.4 kg) nor neonatal BW at birth and 30 h later.[23] Similarly, no differences were observed in the neonatal tissue weights.[23] Except for cobalt, no differences were observed in dam serum trace mineral concentrations at calving. Conversely, calves born to VTM-supplemented dams exhibited greater concentrations of Se and Mo in the liver and Co. and Se in the serum 30 h post-calving.[23] Moreover, VTM treatment increased the concentrations of vitamin A in maternal serum and D in the serum of the calf and the dam.

Maternal VTM supplementation affects transcription factor (TF) regulation and gene expression of the jejunal mucosa in newborn calves

The RNA-Seq approach generated, on average, 30.5 M clean reads per sample (ranging from 21.0 to 38.2 M reads per sample). On average, 94.4% of the total reads were uniquely mapped to the reference genome (Supplementary Table S2). Following post-mapping quality control (QC) by removing not expressed or lowly expressed genes, 15,043 out of 27,607 genes remained in the 14 samples for differential expression analysis.

Using DESeq2, we identified 528 differentially expressed genes (DEGs) between the VTM and CON treatment groups ($P \leq 0.05$ and |Log2FC| ≥ 0.5; Supplementary Table S3). Among them, 231 genes were upregulated, and 297 were downregulated in the VTM neonatal calves (Figure 1A). Most genes consisted of protein-coding (94.49%); however, long noncoding (3.2%), microRNAs (0.19%), and others (2.12%) were also identified. DEGs with the most significant *P*-values were *KCNMB4, ODF3L2, PTPRNA,*

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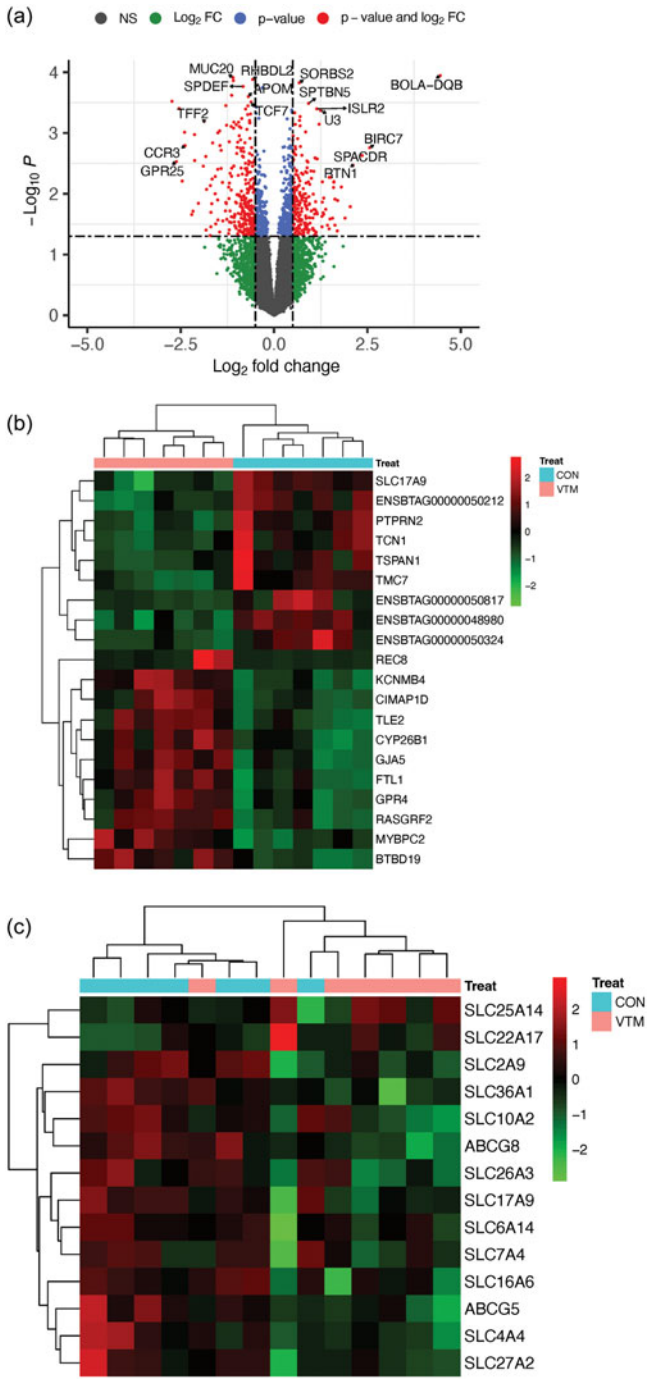

Figure 1. Transcriptomic profile of the jejunal mucosa of neonatal beef heifers born from dams receiving or not receiving vitamin and mineral supplementation (VTM or CON) during gestation. (a) Volcano plot of differentially expressed genes (DEGs) from the jejunal mucosa between VTM vs. CON groups. Each dot represents a gene. The difference in gene expression between the groups is shown as the log2 fold change (*x*-axis). The -log (base 10) of the *P*-value is shown on the *y*-axis. DEGs are color-coded as represented in the legend; (b) Heat map of the top 20 DEGs of the jejunal mucosa of neonatal beef heifers between VTM vs. CON groups; (c) Heat map of genes from the SLC and ABC transporter families identified as DEGs. The heat map colors high and low expression values by red and green, respectively. The expression level of DEGs and groups are color-coded as represented in the legend.

BTBD19, TLE2, TMC7, and *FLT1*. We identified 33 DEGs, which were TFs, including *LBX2, ATOH8*, and *ZBTB7C*.

A heat map of the top 20 DEGs is shown in Figure 1B. To understand the potential effects of VTM treatments on programming genes involved with nutrient transport, we screened the DEG list to identify nutrient transporter-coding genes from the SLC and ABC families. We retrieved 12 genes from the SLC and two from the ABC families (Figure 1C). All these genes were downregulated in the calves born to VTM dams, except for the *SLC25A14* and *SLC22A17*.

Based on the RIF metrics, we identified 98 unique significant TFs out of 1,078 tested as potential regulators of differential gene expression ($P \leq 0.05$). The TFs identified as regulators for the VTM vs. CON groups are reported in Supplementary Table S4. These TFs were grouped into 25 families. Among them, the zinc finger C2H2 and homeobox were over-represented by 41 and 12 TFs, respectively (Supplementary Table S4). From RIF1, the TFs *NFATC1* (*z-score* = − 2.947) and *ONECUT2* (*z-score* = − 2.40) showed the most extreme negative values, whereas *ZSCAN16* (*z-score* = 5.17) and *ZNF274* (*z-score* = 4.66) showed the greatest positive values. Likewise, for RIF2, we found *SMARCAL1* (*z-score* = − 2.48) and *MYC* (*z-score* = − 2.44) as the extreme negative and *E2F3* (*z-score* = 3.42) and *NR2C1* (*z-score* = 3.24) as the extreme positive.

To identify the co-expression profiles between DEGs and RIF genes (TFs), we used partial correlation and information theory.[37] Our network analysis retrieved 43,606 co-expressed pairs ($P \leq 0.05$). To reduce the data dimensionality, we kept 4,783 co-expressed pairs (corresponding to 552 unique genes) with a partial correlation greater than $|0.8|$ ($P \leq 0.05$) (Figure 2; Supplementary Table S5). Among the pairs, we had 92 TFs, from which 65 were also RIF genes (Supplementary Table S5). Based on the network analysis, *ZNF414, ATOH8, NR1H3, NFATC4,* and *LBX2* were the most connected TFs (degrees ranging from 23 to 49; Figure 2).

Maternal VTM supplementation throughout gestation affects genes underlying immune-related pathways in the jejunal mucosa of newborn calves

To identify over-represented pathways and biological processes affected by the DEGs, we performed an over-representation analysis utilizing ClueGO.[39] Significant KEGG pathways (Figure 3) over-represented by DEGs from the VTM vs. CON groups included glycerolipid, glycerophospholipid, and sphingolipid metabolism, which grouped pathways such as cholesterol metabolism, steroid hormone biosynthesis, and fat digestion and absorption. Pancreatic secretion and cGMP-PKG signaling pathways were grouped with several other pathways, including calcium signaling, insulin secretion, thyroid hormone synthesis, and regulation of lipolysis in adipocytes. KEGG pathways consisting of most downregulated genes in the VTM group were fat digestion and absorption and mucin-type O-glycan biosynthesis.

KEGG pathways and BPs related to immune related processes were over-represented (Figure 3). Intestinal immune network for IgA production, cytokine-cytokine receptor interaction, and *Staphylococcus aureus* infection were among the over-represented pathways sharing DEGs. Interestingly, genes underlying the complement and coagulation cascades were mostly downregulated in calves born to VTM dams, including *F5, F2, SERPINF2, SERPINA1, C4BPA, CD55, CFB,* and *C3*. On the other hand, the cytokine-cytokine receptor interaction KEGG pathway showed most genes upregulated in the VTM group (*LIFR, KDR, TNFRSF4, TNFSF18, FLT1,* and *TNFRSF12A*). Figure 3B shows the over-represented BPs, including leukocyte-mediated immunity, leukocyte activation, innate immune response, and defense response ($P \leq 0.05$). Among the underlying genes in most biological processes, we can highlight *HEY2, CACNA1G, IGFBP2, IGFBP6,*

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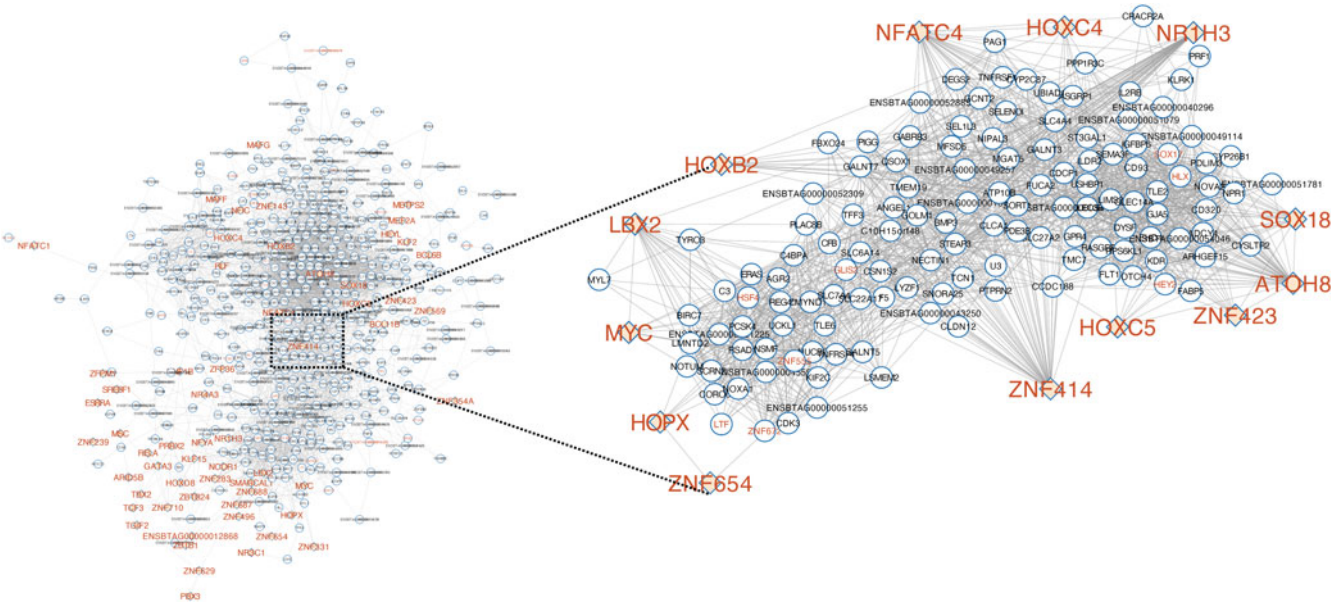

Figure 2. Regulatory network of differentially expressed genes (DEGs) from the jejunal mucosa of neonatal beef heifers born from dams receiving or not receiving vitamin and mineral supplementation (VTM or CON) during gestation. Zoom in on the transcription factors (TFs) with the highest degree and first neighbors. Nodes are DEGs between VTM vs. CON groups and TFs with significant regulatory impact factor (RIF) built through partial correlation information theory (PCIT). Transcription factors are labeled in orange color and those identified as RIFs are represented by a diamond-colored shape. Significantly co-expressed pairs were selected based on a partial correlation value greater than |0.8| ($P \leq 0.05$). Nodes with few connections not linked to the main network are not shown. Cytoscape v.3.9.0 was used for network visualization.

SKAP1, and *KLRK1*. These results shed light on specific pathways and biological processes likely affected by changes in gene expression influenced by maternal VTM supplementation.

To provide a more complete view of the pathways affected by the maternal diet, we performed a Gene Set Enrichment analysis (GSEA) on 15,043 expressed genes, which were ranked based on a combination of P-value and log fold change (*see methods*). We retrieved the top 20 KEGG pathways based on the Normalized Enrichment Score (NES; Figure 4). Top pathways involving upregulated genes in the VTM group included focal adhesion, notch, apelin, and hippo signaling pathways. Additionally, beta-alanine metabolism, cGMP-PKG, Rap1, and MAPK signaling pathways (Figure 4) were over-represented. The *LOC508879, AOC2, FLT1, RASGRF1, KCNMB4*, and *TEAD3* genes were commonly identified among the pathways. Pathways over-represented by downregulated genes (negative NES) in the VTM group mainly included those related to immune response, such as complement and coagulation cascades, natural killer cell-mediated cytotoxicity, and primary immunodeficiency. Furthermore, we identified pathways involved with lipid metabolism, fat digestion and absorption, and hormone biosynthesis. All pathways involved significantly downregulated genes in the VTM group, including *LOC615045, PLA2G12B,* and *PLA2G3.*

Discussion

We found that maternal vitamin and mineral supplementation throughout gestation affected the expression of 528 genes associated with key metabolic pathways and immune functions in the jejunal mucosa of 30 h old newborn calves. Likewise, the VTM treatment influenced the concentration of Co, Se, and Zn in the serum and Se concentrations in the liver of the offspring calves, despite the fact that the maternal VTM supplementation did not influence the BW or organ mass of the calves.[23] The jejunum is a highly plastic tissue that undergoes morphological and physiological changes throughout gestation.[13,44] Thus, it is expected that it is responsive to changes in nutrient availability. These changes can differentially program tissue function, affecting energy utilization and maintenance requirements later in life.[15,21] Using the same calves as the ones in the current study, our previous results investigating tissue oxygen consumption indicate an improved mitochondrial function in the small intestine of VTM calves, as evidenced by the greater mitochondrial efficiency of substrate oxidation and ATP production.[45] Collectively, these findings suggest that the observed transcriptomic changes could influence mitochondrial efficiency or vice versa in response to maternal VTM supplementation.

We have previously shown that maternal vitamin and mineral supplementation combined with the rate of body weight gain (GAIN; moderate (MG) vs. low (LG)) increased the liver mass of heifer fetuses at day 83 of gestation.[6] Likewise, there was a VTM × GAIN interaction for fetal small intestinal weight, where fetuses from VTM-MG dams were heavier than those from VTM-LG, with all other treatments being similar.[6] In a follow-up study with a similar experimental design reported here, we showed that vitamin and mineral supplementation throughout gestation improved the performance of F1 female heifers at weaning and post-weaning development, with a significant impact on BW, feeding behavior, and heifer activity.[7] Interestingly, calf birth BW and body measurements were not different. However, calves born to VTM supplemented dams were heavier than that of CON dams from weaning through 15 months of age,[7] suggesting that there might be a lag time from "insult" to observable effect in offspring performance. Additionally, the effects observed may depend on the interaction with other available nutrients and the dam's nutrient storage, which can buffer the fetus during *in utero* development.[46]

A growing body of evidence has shown that specific vitamins and minerals set up the functioning of the genome through the

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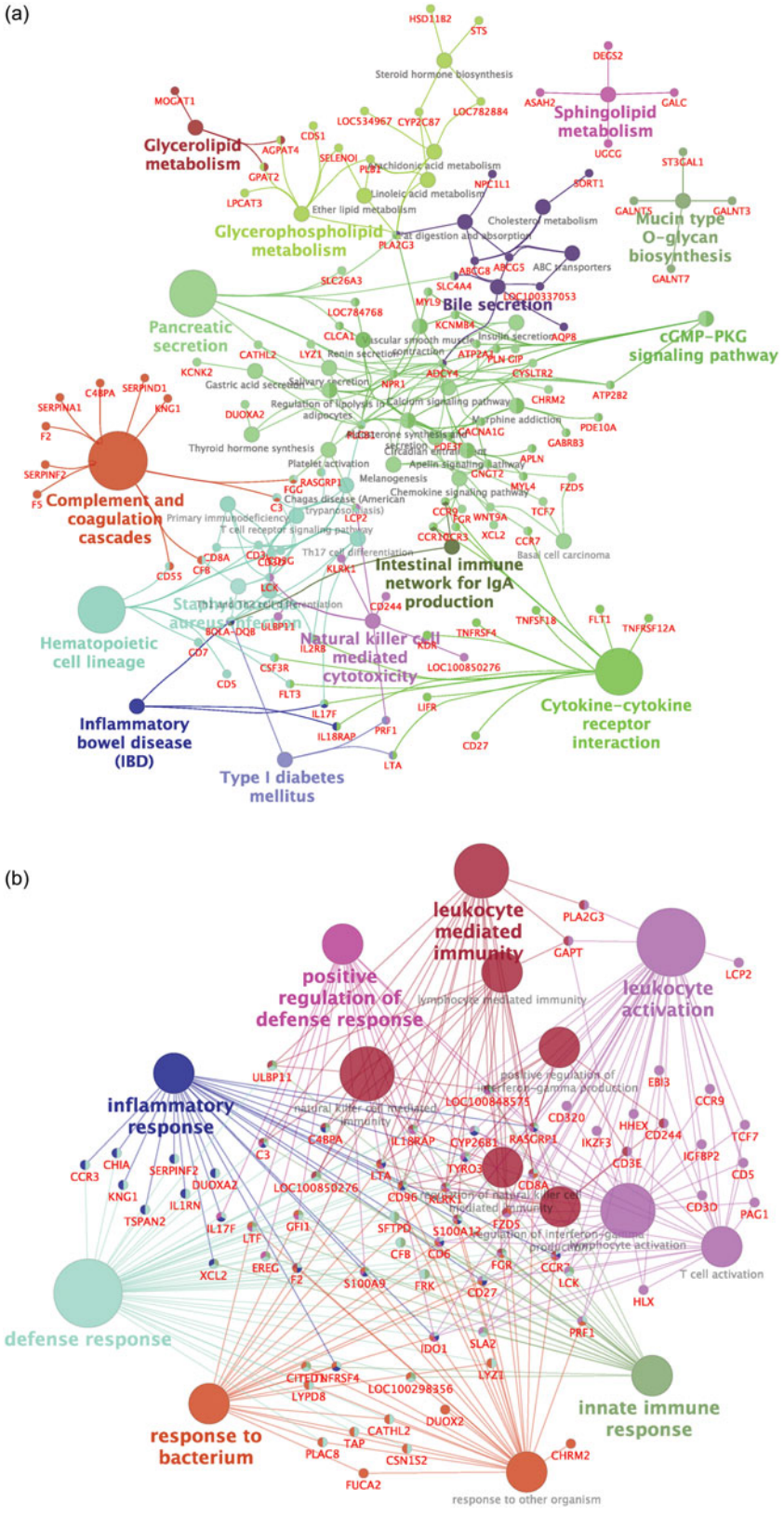

Figure 3. Functional over-representation analysis of differentially expressed genes from the jejunal mucosa of neonatal beef heifers born from dams receiving or not receiving vitamin and mineral supplementation (VTM or CON) during gestation. Network clusters were based on over-represented KEGG pathways (a) and biological processes (b) retrieved from clueGO. Significant terms were taken when $P \leq 0.05$. Functionally related groups partially overlap and are arbitrarily colored. The node size represents the significance of pathway enrichment.

modulation of mineral and vitamin-dependent genes or as enzymatic cofactors.[47] Here, we identified 33 TFs differentially expressed due to maternal diet. Interestingly, 19 were upregulated in the VTM group, which suggests a rewiring of gene networks to modulate the transcriptional output.[48] Our RIF analysis of gene expression retrieved 98 TFs that were differentially co-expressed with DEGs and potential modulators of differential gene expression. *ZNF414, HLX,* and *ATOH8* were the most

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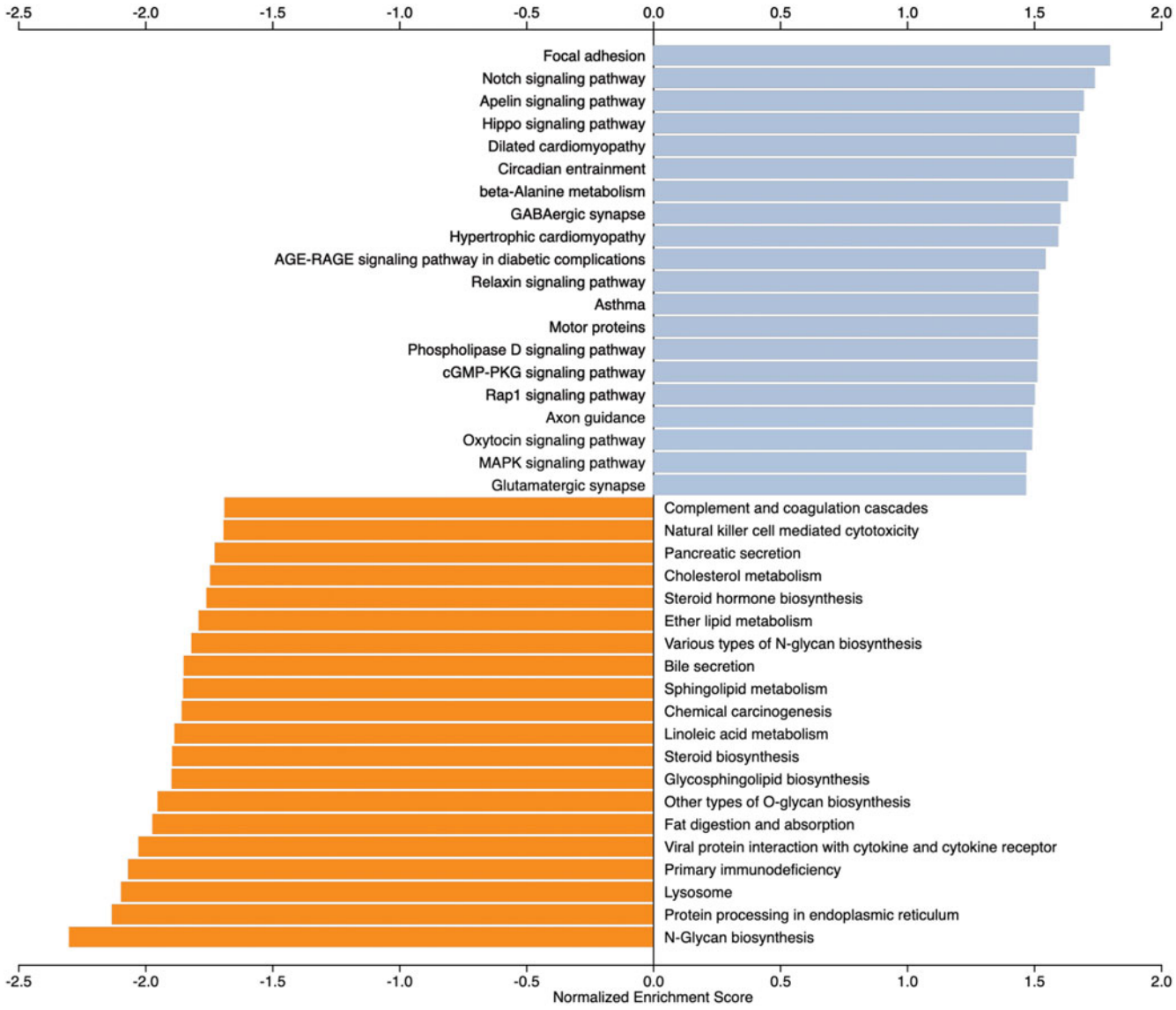

Figure 4. Gene set enrichment analysis-based pathway over-representation of expressed genes from the jejunal mucosa of neonatal beef heifers born from dams receiving or not receiving vitamin and mineral supplementation (VTM or CON) during gestation. The normalized enrichment score (NES) of top enriched (blue bars) and top depleted (orange bars) pathways are based on the comparison of VTM vs. CON calves. Only pathways with a $P \leq 0.05$ and NES $\geq |1.5|$ are shown.

co-expressed TFs in the network. The protein encoded by the *ZNF414* gene has been reported to modulate proliferation, migration, and DNA repair-associated genes.[49] Similarly, the *HLX* TF is required for the development of the enteric nervous system (ENS).[50] Additionally, it plays a key role in visceral organogenesis, including the liver, gall bladder, and gut.[51] The ENS works cooperatively with other cells to control intestinal homeostasis,[52] which includes nutrient digestion and absorption.[53] The changes in nutrient availability may program tissue development *in utero* for postnatal function.[13] Such changes are likely associated with the increased performance post-weaning, as we observed in the contemporary F1 female heifers under a similar experimental approach.[7]

Maternal VTM supplementation throughout gestation altered genes involved with nutrient transport, lipid metabolism, and metabolic genes

Vitamins and minerals act as essential cofactors for enzymes that regulate lipid metabolism, highlighting their importance in animal physiology.[54] Here, we identified DEGs involved with nutrient transport and fat and lipid metabolism pathways, which included cholesterol metabolism, steroid hormone biosynthesis, and thyroid hormone synthesis. Genes from the SLC and ABC families were mainly downregulated in the VTM group. Among them, the *SLC26A3* encodes a key transmembrane chloride ions exchanger.[55] The protein encoded by this gene is localized on the columnar epithelial cells [56] and was reported as important for maintaining the intestinal epithelial barrier function and integrity.[57] Decreased expression of *SLC26A3* leads to upregulation of antimicrobial peptide expression, altering the immunity protection in young mice. Among the upregulated SLC gene members, *SLC22A17* has been associated with the metabolism of drugs and nutrients such as vitamins and flavonoids.[58] The *ABCG5* and *ABCG8* were downregulated in the VTM group. They form an obligate heterodimer that mediates $Mg^{(2+)}$- and ATP-dependent sterol transport in and out of the enterocytes.[59]

We identified other genes and pathways related to lipid metabolism. The *PLCB1* gene, upregulated in the VTM group, is

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part of the phospholipase-C family, which requires minerals like Ca and Zn for optimal activity.[60] The encoded protein is involved with phospholipid hydrolysis and intracellular transduction of extracellular signals.[61] This signaling can influence systemic metabolic processes, given that phospholipase-C activity produces inositol triphosphate (IP3) and diacylglycerol (DAG), molecules that regulate calcium signaling and activate protein kinase C pathways in multiple tissues.[62] Previous studies have associated the *PLCB1* gene with feed efficiency, growth, and carcass traits in multiple species.[63–66] In cattle, *PLCB1* was suggested as a candidate to increase fat thickness and weight gain.[66] The phospholipase D signaling pathway was also over-represented by genes positively ranked in the jejunum tissue based on the GSEA results (genes upregulated in the VTM group). This pathway is involved in fundamental cellular processes that use phosphatidic acid as a secondary messenger[67] activating pathways such as mTOR, which controls several processes, including immune response, survival, proliferation, and migration, to maintain cellular homeostasis.[68] Several pathways underlying fat and lipid metabolism were over-represented by genes negatively ranked within the GSEA analysis (genes downregulated in the VTM group), including fat digestion, absorption, and cholesterol metabolism. From the DEGs acting on these pathways, the *PLA2G3* gene was downregulated in the VTM group. The encoded enzyme hydrolyzes phospholipids to release fatty acids and lysophospholipids.[69] However, the enzyme PLA2G3 also plays a role in immunity-related processes through lipid mediator effects.[69] Likewise, we identified the *LPCAT3* down-regulated in the VTM group. This gene is regulated by nuclear receptors, such as the liver X receptor and peroxisome proliferator-activated receptors, and plays an intricate regulatory role in maintaining cellular lipid balance and function.[70] Modulating lipid metabolism and adipogenesis-related genes could be associated with increased feed efficiency. The observed increased performance of supplemented heifers, particularly the enhanced growth and blood metabolite profiles from weaning through 15 months of age, as reported in Hurlbert et. al.,[7] may be attributed to the modulation of lipid metabolism and adipogenesis-related genes, which are associated with improved feed efficiency. Using a similar experimental design, we have reported that calves born to VTM-supplemented dams had a higher weaning weight (17.5 kgs) and a greater ribeye area compared with CON calves at 209 days of age.[7] Therefore, adequate mineral levels throughout gestation are important for the proper functioning of these genes, as well as enhancing overall performance. Further research, however, at different time points is warranted to determine the extent and persistence of any changes observed as a neonate.

Among the DEGs, the cytochrome P450 family was represented by the downregulated genes *CYP3A74* and *CYP2C87,* which are involved with drug metabolism, synthesis of cholesterol, steroids, and other lipids.[71,72] Furthermore, the *CYP26B1* gene was upregulated in the calves born to VTM dams. The protein encoded by this gene is responsible for retinoic acid (RA) metabolism and homeostasis, intestinal mucosal immune responses, and contributes to vitamin A storage.[73–75] We found that vitamin A was increased in the supplemented dams when compared with the CON group at calving, although the calves had similar circulating concentrations of vitamin A at 30 h after birth.[23] While vitamin A is poorly transferred across the placenta, the colostrum would be the primary source for the calf after birth,[76] which could elicit different transcriptomic responses. Therefore, further studies should investigate the concentrations of fat-soluble vitamins in the colostrum, their storage in the neonatal liver, and their role in developmental programming.

The insulin-like growth factor (IGF) axis is a complex network involving hormones, receptors, and binding proteins,[77] which has a key role in growth, metabolism, and intestinal homeostasis.[78] The IGF axis also mediates growth hormone (GH) actions.[79] Although no differences were observed in the serum concentrations of GH and IGF-1 in the dams and calves,[23] we identified the *IGFBP2* and *IGFBP6* genes upregulated in the VTM group. The IGF binding proteins (IGFBP) are physiologic regulators of the interaction of IGFs with their receptors within the gastrointestinal tract and liver.[79] Changes in the expression of IGFBPs can influence IGF activity by altering its bioavailability and interaction with receptors, though this does not directly equate to changes in IGF concentrations.[79] Additionally, minerals such as Se and Zn were reported as determinants of IGF-1 activity.[80] Similarly, a relationship between IGF-1 concentrations in serum and vitamin D concentrations has been shown.[81] IGFBP2 has been associated with regulating IGF activity in most tissues and organs. Likewise, it can promote transcriptional activation of target genes and contribute to growth and development.[79] Among the regulatory activities, IGF-2 interacts with components of the extracellular matrix, cell surface proteoglycans, and integrin receptors to mediate intracellular signaling.[82,83] Corkins et al. reported that fetal sheep intestinal fibroblasts were responsive to IGF-2 with greater proliferation.[84] The IGF-2 has a role in glucose and lipid metabolism through IGF bioavailability.[83] The knockdown of *IGFBP6* leads to impaired lipid metabolism and decreases cholesterol biosynthesis. Furthermore, the encoded protein has been reported as an important factor in the immune and inflammatory responses.[85,86]

Maternal VTM supplementation throughout gestation downregulate genes involved with immune-related pathways and biological processes in neonatal calves

The intestinal epithelium provides support for nutrients, water uptake, and physical barrier protection.[87] Furthermore, the intestine represents the largest compartment of the immune system.[88] Although developmental adaptations during early life coordinate immune function and development,[22] it has been proposed that the immune system can be programmed *in utero*.[13,44] Carroll et al.[89] reported that exposure to multiple low doses of LPS endotoxin *in utero* had lasting effects on pro-inflammatory and innate immune responses postnatally. The role and importance of minerals in modulating the functioning of the immune system have been discussed in the literature.[90,91] The integrity and functioning of the gut barrier rely on multiple interconnected systems, such as a mucous gel layer, IgA, and junctional proteins.[92,93] We identified focal adhesion, notch, and hippo pathways over-represented by positively ranked genes. These signaling cascades play multiple roles in the maintenance and function of the epithelial cell types.[87] Differential expression of five genes encoding junction proteins included the claudin (*CLDN2* and *CLDN12)* and connexin (*GJA5, GJB3,* and *GJC1*) gene families.

Among the DEGs, the downregulation of *SGPP2* has the potential to positively influence the gut barrier defense.[94] Studies in knockout mice have shown that *SGPP2* deficiency enhances mucosal barrier integrity and barrier function.[94] We also identified several upregulated genes encoding structural proteins, cytoskeleton organization, and tissue development (*GAJ5, MYL7, MYL9, MYO5C, DNAH9, TUBB6, TNNT3,* and *MAST1*). Genes positively ranked within the GSEA analysis (genes upregulated in the VTM

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group) were over-represented in the focal adhesion pathway. These results may suggest the continuous cell turnover, renewal, and differentiation in the intestinal epithelium due to significant changes in enteral nutrition. The *CATHL2* gene was reported to exhibit antimicrobial activity against various pathogens,[95] and was identified as downregulated in the VTM group. While primarily associated with immune function, peptides like CATHL2 may indirectly influence gut barrier integrity by modulating the microbiota and defending against pathogenic invasion, possibly impacting aspects of metabolism and feed efficiency indirectly through effects on gut microbiota.[96] From the same calves used in the current study, we have reported that the offspring microbial community in the rumen (ruminal fluid and tissue-associated microbiota) was affected by the maternal vitamin and mineral supplementation.[97] Such changes were related to the community structure (ruminal fluid), diversity (ruminal fluid and tissue), and composition, suggesting the role of maternal diet during fetal development on the initial microbial colonization of the neonatal calf gut.[97] Research using the intestinal epithelial cells from mice reared in the presence or absence of microbiota has shown changes in the host gene transcription. In cattle, host-microbial interactions in the rumen were associated with genes underlying immune system-related biological processes. However, the regulatory mechanism between this interaction and the role of nutrients remains unclear. Thus, understanding the interplay between maternal nutrition and the offspring's intestinal function provides opportunities to positively program the offspring outcomes.

Neonates are immunocompetent at birth and rely on the passive transfer of immunoglobulins (Ig) from maternal colostrum to acquire protection.[22] We identified multiple biological processes associated with the immune system and inflammatory responses, including the intestinal immune network for the IgA production pathway. The IgA antibodies mediate the humoral adaptive immune response at mucosal surfaces, serving as the first line of defense against microorganisms.[98,99] Overall, the underlying genes acting on immune-related biological processes and pathways were downregulated in the VTM calves, except for those on the cytokine-cytokine receptor interaction pathway, in which six out of eight genes were upregulated. The GSEA analysis showed the same downregulation pattern with the pathways over-represented by negatively ranked genes. We have reported that among 15 tested serum cytokines, the concentration of IP-10 was increased while IL-4 and IL-17A tended to be decreased in VTM calves compared with CON.[97] Among the interleukin-related genes, we found *IL2RB, IL17F, IL1RN, IL18RAP* downregulated and *ILDR2* upregulated in the VTM group. It is possible that the pro-inflammatory and anti-inflammatory cytokines produced by the intestine would affect their levels in the blood. However, the panel used was limited to only 15 cytokines. Further research is warranted to fully understand the effects of minerals and vitamins on immune response.

The list of upregulated DEGs with regulatory impact effects on differential expression included the *ATOH8* gene. This TF acts in fundamental processes and functions as both a transcriptional activator and a repressor.[100] Additionally, *ATOH8* acts as a regulator of intestinal microfold cells (M cells), which are responsible for immune sensing in response to intestinal pathogens/antigens.[101] The M cells are found in the epithelium, covering mucosa-associated lymphoid tissues.[102] In addition to the M cells, the mucosal surface is protected by physical shields composed of mucin and glycocalyx layers and chemical barriers such as antigen-specific IgA.[103] We identified several chemokines downregulated in the VTM calves, except for the *CCR10*, which was upregulated. The protein encoded by the *CCR10* gene regulates the intestinal IgA response and memory maintenance.[104] Chemokines and chemokine receptors are key players in attracting lymphocytes and leukocytes to lymphoid tissues.[105] We found leukocyte-mediated immunity, leukocyte activation, and lymphocyte-mediated immunity among the over-represented biological processes.

The immune system is a complex, tightly regulated, cooperative network that requires a balance between positive and negative immune modulators.[106,107] Nutrients, such as vitamins and minerals, play unique regulatory roles in the programming of the immune system during *in utero* fetal development, with potential effects on the postnatal immune response.[90] Therefore, the implications of maternal nutrition on turning on or off these genes warrant further investigation.

Our results highlight that maternal VTM supplementation throughout gestation can have a significant impact on many important genes associated with tissue structure, nutrient transport, and immune system pathways. Although individual changes in gene expression may not have major phenotypic impacts, our analysis showed changes in several TFs, which can have downstream effects on regulatory pathways. Additionally, our in-silico analysis pointed out key transcriptions factors that are likely modulating the differences in gene expression. While this study provides novel insights into the potential programming of jejunum development at birth, it has some limitations. Our study relied on a single-time-point analysis, which limits the ability to capture the dynamic changes in gene expression. Thus, to provide a comprehensive understanding of gene expression dynamics and interactions, longitudinal studies at multiple pre and postnatal time points are necessary to determine whether the observed gene expression changes persist and lead to lasting physiological effects. Additionally, our study was limited to the jejunal mucosa, and further research is needed to investigate the impact of maternal supplementation on other tissues and organs integral to growth and metabolism. The long-term implications in animal health due to the downregulation of immune-related genes in the supplemented group warrant further research. Likewise, research is needed to determine the mechanisms involved with fetal programming and whether the extensive alterations in immune-associated transcript abundance at the neonatal timepoint are sustained into further postnatal life and culminate into alterations in long-term outcomes on cattle herd health.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S2040174425000157.

Data availability statement. All relevant data are within the paper and its Supplementary Information files. All additional datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

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Ethical standard. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of animals and have been approved by the North Dakota State University Institutional Animal Care and Use Committee (#A21047).

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