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Multiomics reveals blood differential metabolites and differential genes in the early onset of ketosis in dairy cows

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ABSTRACT

Ketosis—a metabolic state characterized by elevated levels of ketone bodies in the blood or urine—reduces the performance and health of dairy cows and causes substantial economic losses for the dairy industry. Currently, beta-hydroxybutyric acid is the gold standard for determining ketosis in cows; however, as this method is only applicable postpartum, it is not conducive to the early intervention of ketosis in dairy cows. In this study, the sera of dry, periparturient, postpartum ketotic, and healthy cows were analyzed by both transcriptomics and metabolomics techniques. Moreover, changes of gene expression and metabolites were observed, and serum physiological and biochemical indexes were detected by ELISA. The purpose was to screen biomarkers that can be used to detect the incidence of dry or periparturient ketosis in cows. The results showed that ketotic cows had increased levels of glycolipid metabolism indexes, oxidizing factors, and inflammatory factors during dry periods and liver damage, which could be used as early biomarkers to predict the onset of ketosis. Transcriptomic results yielded 20 differentially expressed genes (DEGs) between ketotic and healthy cows during dry, peripartum, and postpartum periods. GO and KEGG enrichment analyses indicated that these DEGs were involved in amino acid metabolism, energy metabolism, and disease-related signaling pathways. The metabolomics sequencing results showed that ketotic cows mainly showed enrichment in tricarboxylic acid cycle, butyric acid metabolism, carbon metabolism, lysine degradation, fatty acid degradation, and other signaling pathways. Metabolites differed between ketotic and healthy cows in dry, pre-parturition, and post-parturition periods. Combined transcriptomics and metabolomics analyses identified significant enrichment in the glucagon signaling pathway and the lysine degradation signaling pathway in dry, periparturient, and postpartum ketotic cows. PRKAB2 and SETMAR—key DEGs of the glucagon signaling pathway and lysine degradation signaling pathway, respectively—can be used as key marker genes for determining the early onset of ketosis in dairy cows.

1. Introduction

Ketosis is one of the major metabolic diseases in periparturient dairy cows [29]. The main cause of ketosis is a negative energy balance caused by insufficient dietary intake in the post-perinatal period that does not meet the cow's own maintenance energy requirements and milk production needs. If metabolic disorders induced by this negative energy balance are not resolved in time, cows will eventually develop either subclinical ketosis or clinical ketosis [26]. The main clinical symptoms of ketosis in dairy cows are loss of appetite, decreased milk production, weight loss, hypoglycemia, and high blood ketone levels [17]. The prevalence of ketosis in dairy cows has been estimated to range from 6.9 % to 43 % [11,39]. During reproduction, cows with ketosis may develop insulin resistance and oxidative stress [58], which can substantially reduce milk production and reproductive efficiency; it can also increase the incidence of abdominal displacement, lameness, and metritis [26,32], which undermines the healthy development of the dairy industry. In addition, the milk yield of ketotic cows is 5–7 kg/d lower compared to that of healthy cows in the same period [27]. The economic losses (e.g., incurred by diagnosis, treatment, loss of milk yield, loss of reproduction, and loss through mortality) of primiparous and lactating cows with ketosis are $134 and $111, respectively [28], which results in huge losses to the breeding industry.

Omics techniques are increasingly being used to study the molecular

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mechanisms underlying complex disease pathogenesis in cattle. Metabolomics has become an attractive analytical tool with highly accurate predictive and diagnostic capabilities in research related to ketosis and mastitis in dairy cows [8,59], such as heat stress [36], mastitis [9], hoof disease [37], and lipid metabolomics disorders [15]. Current transcriptomic and metabolomic studies related to the pathogenesis of ketosis mainly focused on pre-perinatal and post-perinatal periods. Loiklung et al. [62] used omics techniques to screen cholesterol esters, phospholipids, free fatty acids, and triacylglycerol in milk as biomarkers for the diagnosis of the onset of ketosis in dairy cows. Using transcriptomics and metabolomics technology, Wu et al. [49,50] tested perinatal ketotic cows and found five marker genes and two marker metabolites to be associated with the onset of perinatal ketosis. Other techniques can also be used to predict the onset of ketosis; for example, bioanalytical thin-layer chromatography and gas chromatography can use four fatty acids in milk as biomarkers to diagnose the onset of ketosis in dairy cows (16). In addition, a combination of RNA-Seq with genome-wide association signaling techniques can also be used to screen for genes associated with ketosis (17). Transcriptomic and metabolomic studies related to the occurrence of ketosis in dairy cows mainly focus on the prenatal and postnatal periods of dairy cows, but studies on the dry milk period of dairy cows are rare. There are few reports on the key genes and biomarkers of the early onset of ketosis in dairy cows that integrate the dry milk period, pre-perinatal period, and post-perinatal period of dairy cows. Therefore, ELISA was used to detect the changes of physiological and biochemical indexes in the blood of both ketotic and healthy cows during dry, pre-perinatal, and post-perinatal periods. Transcriptomic and metabolomic sequencing technology was used to explore the changes of gene expression and metabolites in ketotic cows and healthy cows during these periods. Further, the diagnostic factors for early onset of ketosis in cows were explored to provide biomarker reference and technical support for the early diagnosis of ketosis in cows.

2. Materials and methods

2.1. Experimental animals

This experiment was conducted on a large dairy farm (>4200 cows) in Ningxia, China, from June 15 to August 25, 2022. All experiments were approved by the Animal Ethics Committee of Ningxia University (No. 22–72). The experimental procedures were carried out in strict accordance with approved guidelines and regulations. Dairy cows were housed in loose pens, were fed total mixed rations three times per day, at 06:00, 12:00, and 18:00, and were free to eat and drink. Fifty-seven healthy cows with similar parities (3–5 parities) and similar delivery dates were selected for the experiment. After a one-week adaptation period, 10 mL of tail root venous blood was collected every morning (06:00) during dry period (40–60 days before delivery), pre-perinatal period (15–21 days before delivery), and post-perinatal period (5–10 days after delivery). The content of beta-hydroxybutyric acid (BHBA) in the blood of each cow was measured by a blood ketone meter (FreeStyle Optium Neo, Abbott Diabetes Care, USA). Cows with a BHBA <1.2 mmol/L can be considered as healthy cows, and cows with a BHBA ≥ 1.2 mmol/L can be considered as ketotic cows [24]. Cows were culled if they developed mastitis, stomach displacement, endometritis, bloody milk, and other symptoms throughout the experimental period. A total of six ketosis cows were screened from the dry period, pre-partum and post-partum periods during the experiment. These six cows, which suffered from ketosis in all three periods and had the same number of births (three births), which were denoted as the dry-period ketosis cows (DKC), the pre-peripartum ketosis cows (PKC), and the post-peripartum ketosis cows (KC), respectively. Also according to the level of BHBA <1.2 mmol/L of perinatal cows, six healthy cows with the same parity (three parities) were screened, which were dry healthy cows (DHC), pre-perinatal healthy cows (PHC), and post-perinatal healthy cows (HC).

2.2. Blood sample collection and processing

A total of 10 mL of tail venous blood from healthy cows ($n = 6$) and ketotic cows ($n = 6$) was collected using a vacuum blood collection vessel containing EDTA K2 anticoagulant. The samples were gently mixed upside down, transferred to a sterile frozen storage tube within 1 h, then placed in liquid nitrogen, and brought back to the laboratory for storage at −80 °C for the determination of physiological and biochemical indexes. A total of 10 mL of the above dairy cow tail venous blood was also collected by vacuum sampling vessel without anticoagulant. The blood sample was coagulated at room temperature for 30 min and centrifuged at 3000 ×*g* at 4 °C for 15 min. The supernatant was collected and divided into sterile freezer storage tubes, then placed in liquid nitrogen, and brought back to the laboratory for storage at −80 °C for transcriptome sequencing. A total of 10 mL of tail venous blood from healthy cows ($n = 6$) and ketotic cows ($n = 6$) was also collected using a vacuum blood collection vessel containing EDTA K2 anticoagulant, which was left at room temperature for 30 min, and then centrifuged at 3000 ×*g* at 4 °C for 15 min. The supernatant was collected and divided into aseptic freezer tubes, which were then placed into liquid nitrogen and brought back to the laboratory for storage at −80 °C for metabolome sequencing.

2.3. Analysis of physiological and biochemical indicators

The contents of alanine aminotransferase (ALT,) (No.M0502A), glutamine aminotransferase (AST) (No.M0503A), triglyceride (TG) (No. RXJ1600917B), glucose (GLU) (No.M1501A), BHBA glutathione peroxidase (GSH-Px), (No.M0304A) malondialdehyde (MDA) (No. M0106A), and superoxide dismutase (SOD) (No.M0101A) were determined by relevant kits (Suzhou Monohydro Bio-pharmaceutical Science and Technology Co., Ltd., China). The contents of inflammatory factors interleukin-6 (IL-6) (No.RX1600853B), interleukin-8 (IL-8) (No. RX1600852), Band tumor necrosis factor (TNF-α) (No.RX1600738B) were determined by an ELISA kit (Zhanxin Biotechnology Co., Ltd., China).

2.4. Transcriptome analysis

2.4.1. RNA extraction and library construction

Total RNA was extracted from the collected whole blood samples of dairy cows. A transcriptome library was constructed after the integrity, concentration, and purity of RNA had been tested and qualified; sequencing was performed using an Illumina HiSeq sequencing platform. The original data were filtered, spliced sequences and low-quality reads were removed, and high-quality clean data were obtained. Sequence comparison with the reference genome Bos_taurus.ARS-UCD1.2.dna.toplevel.fa was performed to obtain map data. The quality of the sequencing library was evaluated by examining both the length and randomness of inserted fragments. Transcriptome sequencing was performed by Baypur Spectrum Biotechnology Ltd. (Shanghai, China).

2.4.2. Differentially expressed gene screening and pathway analysis

Differentially expressed genes (DEGs) were analyzed using the read count matrix and DESeq 2.0 software. $\text{Log2}|\text{FoldChange}| \geq 1$ and $P < 0.05$ were used as screening criteria for DEGs. A volcano map and DEGs were plotted using the ggplot2 package in R (v 3.6.2). GO functional analysis (http://www.geneontology.org/) and KEGG pathway annotation (http://www.genome.jp/kegg/) of DEGs were performed using clusterProfiler in R (v 3.10.1).

2.4.3. qPCR validation

Seven genes were randomly selected from DEGs and the sequencing results were verified by qPCR. qPCR primers were designed using Primer Premier 5.0 software (Table 3). For all 18 RNA samples used for RNA-SEQ analysis, cDNA was prepared, the gene expression level was

Table 3
Primer information for qPCR validation of DEGs.

Gene Name	Gene Bank	Primer Sequence(5′—3′)	Product Length(bp)	Temperature
IFT88	NM_001110786.3	F- GCTCTGCGTTTGACCCTC R- AAAGAGAGGTTGGTCGCAGC	1048	60 °C
PRKAB2	NM_001192328.1	F- AGCCATAATGACTTTGTTGCC R- AATAACTTGGAGAAGGTGAGGA	327	60 °C
SETMAR	NM_001075508.1	F- GTGCACTGAGCCAGTTTTCG R- CCAGCCTTTGTGATCCGTCT	124	60 °C
RPS10	NM_001034716.2	F- CCAACGAGGGCATCCAGTAT R- CACGACCAAATCCGCCTCTA	261	60 °C
PDK2	NM_001159481.1	F- GCTCTTTGACGGCAGAACCA R- GCTGTTTGGAGTTGGATGCG	182	60 °C
TMEM177	NM_001192060.1	F- CTATGCCTTCTCCACCGACT R- CGTGTAGGGTAGGTGTTTGATG	232	60 °C
ATG4D	NM_001099146.1	F- TGTATCTCGCCTATGGCTCAC R- GGGCAGGAAATGAAGCAGAA	142	60 °C
GAPDH	NM_001034034.2	F-GGCATCGTGGAGGGACTTATG R-GCCAGTGAGCTTCCCGTTGAG	186	60 °C

normalized to GAPDH, and the relative gene expression levels were calculated by the $2^{-\triangle\triangle ct}$ method [23]. Three biological replicates and three technical replicates were conducted per sample.

2.5. Metabolome analysis

2.5.1. Sample preparation and metabolite extraction

Plasma samples were separated in a 4 °C injector using the 1290 Infinity LC ultra-high performance liquid chromatography system (Agilent Technologies Co., Ltd.), and positive and negative ion patterns were detected for each sample by electrospray ionization. The ultra-high performance liquid chromatography samples were analyzed by Triple-TOF5600 mass spectrometer (AB SCIEX, USA). The extraction, identification, and quantitative analysis of metabolites were performed by Shanghai Baypur Biotechnology Co., Ltd. (Shanghai, China).

2.5.2. Metabolome data processing

The structures of metabolites were identified by MassBank, HMDB, and other self-established metabolite standard libraries and public databases. The metabolite information was extracted by orthogonal partial least squares discriminant analysis (OPLS-DA). Metabolites with variable importance in projection ≥ 1 and univariate statistical analysis $P < 0.05$ were selected as differentially accumulated metabolites (DAMs). These DAMs were annotated using KEGG compound data (http://www.kegg.jp/kegg/compound/) and KEGG pathway database (http://www.keggjp/kegg/pathway.html). p values were adjusted for false discovery rate in multiple comparison testing. $p < 0.05$ was considered statistically significant.

2.6. Combined analysis of transcriptome and metabolome

According to the results of DAMs and DEGs analyses, the KEGG enrichment results of DEGs and DAMs in dry, pre-perinatal, and post-perinatal periods were analyzed, and a significant KEGG bubble map was plotted.

2.7. Data analysis

Statistical analysis was performed using GraphPad Prism 8.0.2, and data were analyzed by unpaired two-way ANOVA. $P < 0.05$ indicates a significant difference, denoted by *; $P < 0.01$ indicates a significant difference, denoted by **. Three technical repeats were performed for each sample. Transcriptomic and metabolomic data were analyzed using the min-max normalization method of TBtools [5].

3. Results

3.1. Liver function, glycolipid metabolism, redox indicators, and inflammatory factor content

According to the results of ELISA, the contents of ALT, AST, TG, BHBA, NEFA, and GLU in serum of both healthy and ketotic cows are shown in Fig. 1. The contents of ALT (Fig. 1A, $P < 0.01$), TG (Fig. 1C, $P < 0.01$), and GLU (Fig. 1F, $P < 0.01$) in the blood of DKC were significantly higher than those in DHC; the contents of ALT (Fig. 1A, $P < 0.05$), TG (Fig. 1C, $P < 0.01$), and NEFA (Fig. 1E, $P < 0.01$) in the blood of PKC were significantly higher than those in PHC; the contents of ALT (Fig. 1A, $P < 0.01$), AST (Fig. 1B, $P < 0.01$), TG (Fig. 1C, $P < 0.01$), BHBA (Fig. 1D, $P < 0.05$), and NEFA (Fig. 1E, $P < 0.01$) in the blood of KC were significantly higher than those of HC; the contents of GLU (Fig. 1F, $P < 0.01$) in the blood of KC were significantly lower than those of HC.

The determination results of SOD, GSH-Px, and MDA in blood of healthy and ketotic cows are shown in Fig. 2. The MDA content in blood of DKC was significantly higher than that of DHC (Fig. 2C, $P < 0.05$); the activities of SOD (Fig. 2A, $P < 0.01$) and GSH-Px (Fig. 2B, $P < 0.01$) in blood of KC were significantly lower than those of HC, while the MDA content (Fig. 2C, $P < 0.01$) was significantly higher than that of KC.

The results of IL-6, IL-8, and TNF-α in blood of ketotic and healthy cows during the dry, pre-perinatal, and post-perinatal periods are shown in Fig. 3. The results show that the IL-6 levels in DKC, PKC, and KC were significantly higher than those in DHC, PHC, and HC (Fig. 3A, $P < 0.01$), respectively. The contents of IL-8 (Fig. 3B, $P < 0.01$) and TNF-α (Fig. 3C, $P < 0.01$) in the blood of KC were significantly higher than those of HC.

Significance analysis of the healthy and ketosis groups in the three periods separately revealed that MDA (Supplementary Fig. 10G) content significantly increased and SOD (Supplementary Fig. 10H) significantly decreased in healthy cows during the late periparturient period, and ALT (Supplementary Fig. 10A),AST (Supplementary Fig. 10B), BHBA (Supplementary Fig. 10D), and NEFA (Supplementary Fig. 10E) contents increased significantly in the late periparturient period of cows with ketosis and GLU (Supplementary Fig. 10F) levels decreased significantly.

3.2. Gene expression, clustering, and differentially expressed gene screening

A total of 850 million reads were identified by transcriptome sequencing, and the share of clean reads among raw reads was 94.52–95.56 %. The alignment rate exceeded 84 %, Q20 $\geq$ 96.59, and Q30 $\geq$ 92.2, indicating that the sequencing data were reliable and could be used for subsequent analysis. The reference sequence alignment table

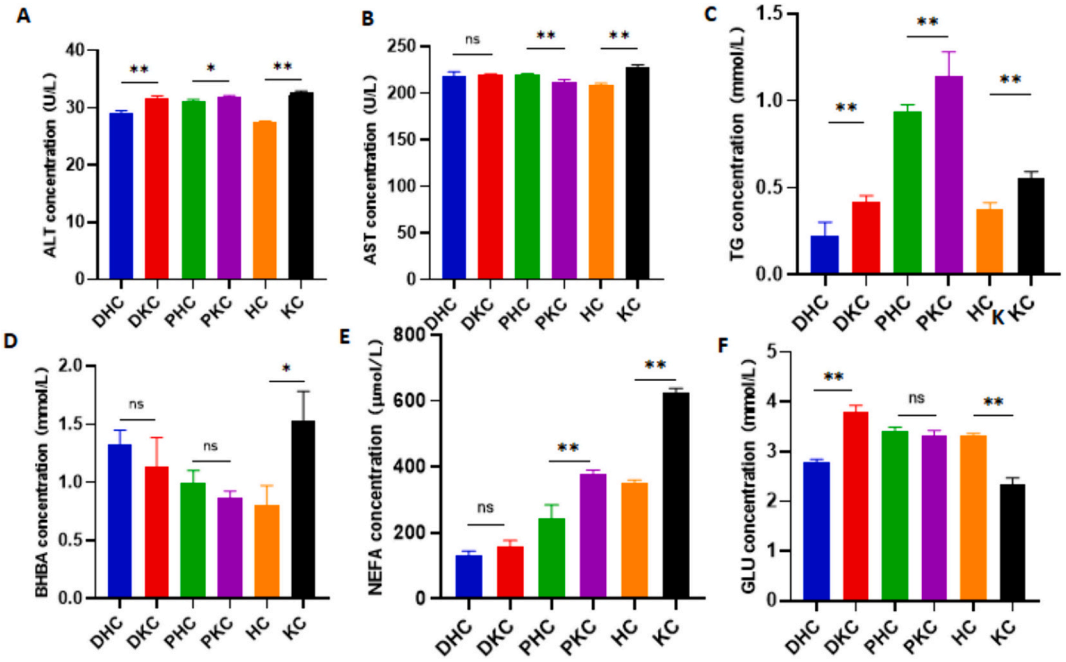

Fig. 1. Metabolic content of hepatic lipids in the blood of healthy and ketotic cows during DHC, DKC, PHC, PKC, HC, and KC.
A and B: ALT and AST levels in DHC, DKC, PHC, PKC, HC, and KC. C and D: TG and BHBA levels in DHC, DKC, PHC, PKC, HC, and KC. E and F: NEFA and GLU levels in DHC, DKC, PHC, PKC, HC, and KC. DHC: Healthy cows in the dry period. DKC: Ketotic cows in the dry period. PHC: pre-partum healthy cows, PKC: peripartum ketotic cows, HC: peripartum healthy cows, KC: late peripartum ketotic cows. ns represents no significant difference, $P > 0.05$. * represents significant difference at $P < 0.05$. ** represents significant difference as $P < 0.05$. Same as below.

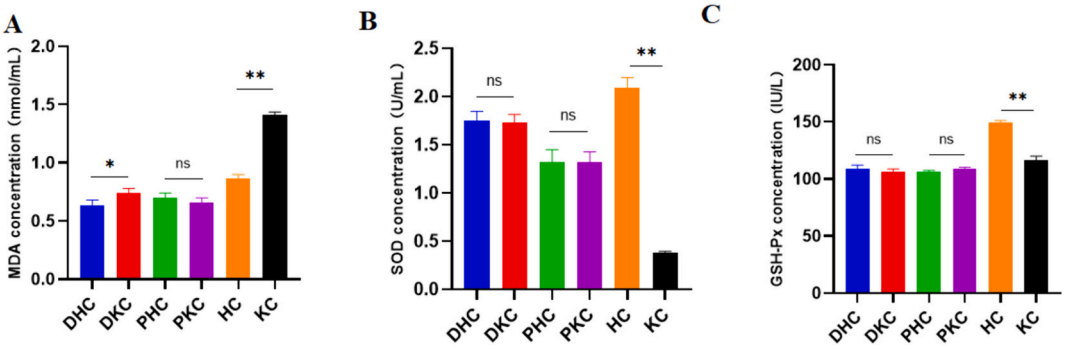

Fig. 2. Oxidized reduced causative agent content in healthy and ketotic cows in DHC, DKC, PHC, PKC, HC, and KC.
A: MDA levels in DHC, DKC, PHC, PKC, HC, and KC. B: SOD levels in DHC, DKC, PHC, PKC, HC, and KC. C: GSH-Px levels in DHC, DKC, PHC, PKC, HC, and KC.

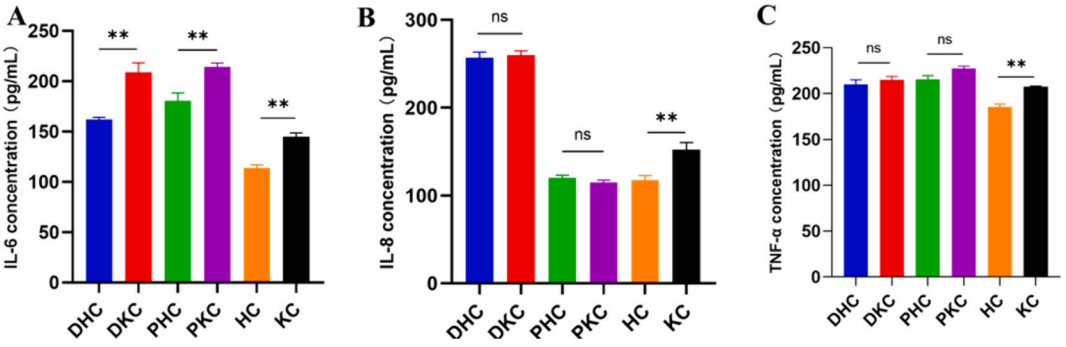

Fig. 3. Levels of inflammatory factors in healthy and ketotic cows in DHC, DKC, PHC, PKC, HC, and KC.
A: IL-6 levels in DHC, DKC, PHC, PKC, HC, and KC. B: IL-8 levels in DHC, DKC, PHC, PKC, HC, and KC. C: TNF-α levels in DHC, DKC, PHC, PKC, HC, and KC.

Table 1
Shows the comparison of reference sequences.

Sample	Raw Reads.	Clean Reads	Clean Reads(%)	Total_Mapped	Q20(%)	Q30(%)
DHC-1	42,422,074	40,538,644	95.56	36,377,481 (89.74 %)	96.68	92.46
DHC-2	49,213,356	46,692,638	94.87	41,368,532 (88.60 %)	96.87	92.75
DHC-3	44,779,842	42,478,384	94.86	38,026,387 (89.52 %)	96.98	92.95
DKC-1	51,953,510	49,303,302	94.89	43,010,936 (87.24 %)	96.94	92.3
DKC-2	49,768,684	47,189,032	94.81	43,003,741 (91.13 %)	96.96	92.52
DKC-3	40,938,600	38,722,116	94.58	35,601,405 (91.94 %)	96.97	92.5
PHC-1	42,823,752	40,613,758	94.83	36,495,203 (89.86 %)	96.9	92.64
PHC-2	43,282,430	41,010,738	94.75	37,233,327 (90.79 %)	96.77	92.01
PHC-3	47,243,170	45,010,108	95.27	40,868,025 (90.80 %)	96.59	92.2
PKC-1	52,945,958	50,086,980	94.6	42,828,563 (85.51 %)	96.85	92.32
PKC-2	47,151,398	44,594,096	94.57	38,925,549 (87.29 %)	97.15	92.85
PKC-3	54,382,596	51,450,642	94.6	47,443,552 (92.21 %)	97.07	92.62
HC-1	41,911,560	39,910,346	95.22	36,693,086 (91.94 %)	96.63	92.19
HC-2	50,075,904	47,429,038	94.71	43,275,276 (91.24 %)	96.91	92.55
HC-3	44,504,124	42,253,646	94.94	35,551,890 (84.14 %)	96.64	92.08
KC-1	43,716,946	41,581,752	95.11	37,574,362 (90.36 %)	96.99	92.98
KC-2	54,217,558	51,308,082	94.63	46,522,735 (90.67 %)	96.98	92.48
KC-3	46,162,894	43,635,446	94.52	40,177,613 (92.08 %)	97.13	92.55

is shown in Supplementary Table 1.

The closer the correlation coefficient is to 1, the higher the similarity of expression patterns between samples. Analysis of the correlation of gene expression levels in all samples in this experiment showed that the sample correlation coefficients of DHC, DKC, PHC, PKC, HC, and KC ranged from 0.6 to 1, indicating that the correlation coefficients between samples in this test were strong and expression patterns were relatively similar (Supplementary Fig. 1A). Principal component analysis of metabolites was performed to identify the overall variability between and within sample groups. It was found that DHC and DKC, PHC and PKC, as well as HC and KC were all relatively separated, indicating that the samples were well grouped. The samples within groups are better clustered together, indicating very little within group variation (Supplementary Fig. 1B).

DESeq software was used to screen for significant DEGs, and the screening condition was that the expression differentially expressed multiple $|\log2FoldChange| > 1$, and the significance $p < 0.05$. A volcano map of DEGs was drawn as shown in Fig. 4. The screening results indicated that there were 3290 DEGs between DHC and DKC (Fig. 4A), 2122 of which had significantly higher relative expression levels in the DKC group than DHC, and 1168 DEGs in the DKC group had significantly lower relative expression levels than DHC. This result suggests that there were differences in transcription levels between healthy cows and ketotic cows from the dry stage. A comparison between PHC and PKC showed that the relative expression levels of 344 DEGs in PKC were higher than those in PHC, and the relative expression levels of 276 DEGs in PKC were lower than those in PHC (Fig. 4B). Comparison of KC and HC showed that 228 DEGs were significantly upregulated and 93 DEGs were significantly downregulated (Fig. 4C; Supplementary Fig. 2).

To validate the RNA-Seq data, seven DEGs were selected for qPCR analysis. The results showed consistent gene expression trends of RNA-Seq and qPCR (Supplementary Fig. 4).

3.3. GO enrichment analysis and KEGG enrichment analysis of differentially expressed genes

To explore the functions of DEGs between ketotic and healthy cows at different physiological stages, GO enrichment analysis and KEGG signaling pathway enrichment analysis were performed on DEGs (Fig. 5). The top 20 GO and KEGG pathways with the most significant enrichment were selected. The results showed that the GO pathways were mainly associated with primary metabolism, nitrogen compound metabolism, organic metabolism, and cell metabolism (Supplementary Fig. 3A). The significantly enriched KEGG signaling pathways were mainly associated with cell cycle and aging, oxidative phosphorylation, non-alcoholic fatty liver disease, diabetic cardiomyopathy, and other disease-related signaling pathways (Fig. 5A). The GO items that were significantly enriched between PHC vs PKC were primary metabolism, nitrogen compound metabolism, organic metabolism, and cell metabolism (Supplementary Fig. 3B). Significantly enriched KEGG signaling pathways were β-alanine metabolism, inflammatory pathways, IL-17 signaling pathways, physiological and pathological pathways, CGMP-PKG signaling pathway, cell signaling pathway, TNF signaling pathway, and ErbB signaling pathway (Fig. 5B). The DEGs between KC and HC were mainly enriched to GO term (Supplementary Fig. 3C), and significantly enriched KEGG signaling pathways were mainly metabolic

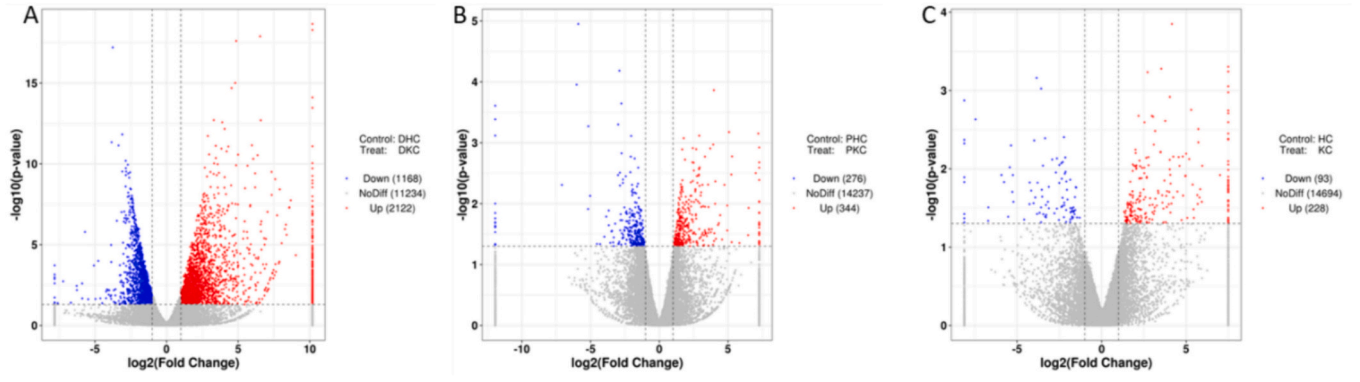

Fig. 4. Volcanic map of DEGs in DHC, DKC, PHC, PKC, HC, and KC.
A: DHC and DKC. B: PHC and PKC. C: HC and KC.

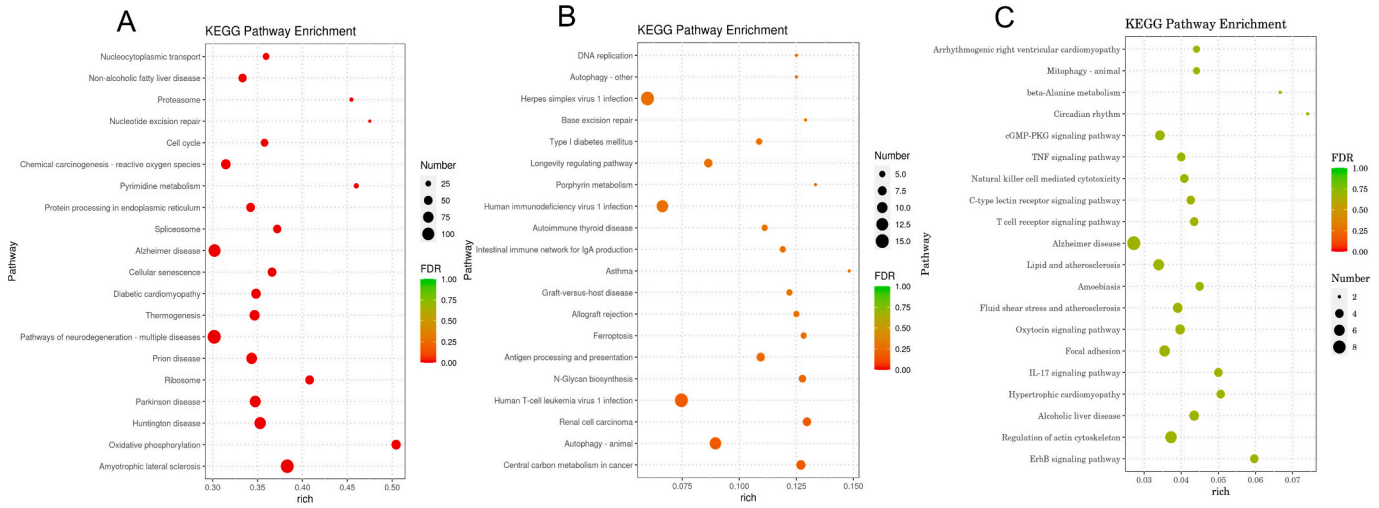

Fig. 5. Enrichment maps of KEGG pathway for DEGs in DHC, DKC, PHC, PKC, HC, and KC.
A: DHC and DKC. B: PHC and PKC. C: HC and KC.

pathways, lipid and atherosclerotic pathways, amebiasis, hypertrophic cardiomyopathy, alcoholic liver disease, and other disease-related pathways (Fig. 5C).

3.4. Transcriptome analysis of shared differentially expressed genes and enrichment pathways

A total of 20 DEGs were identified, including BRWD1, REL, ENSBTAG00000050908, SETMAR, FAM91A1, GK, JMY, HNRNPLL, PRKAB2, DPY19L4, SLC16A12, GCNT4, CCNT1, TBL1XR1, SRGN, TBC1D15, MMS22L, PTP4A3, AGFG1, and ENSBTAG00000039980 (Fig. 6A). The results of GO and KEGG analyses showed that the above DEGs enriched on GO were mainly related to protein kinase complex, biosynthesis process of glycerol 3-phosphate, and triglyceride metabolism (Fig. 6B); the DEGs enriched on KEGG signaling pathways mainly included glucagon signaling pathway, AMP-activated protein kinase (AMPK) signaling pathway, and triglyceride metabolism (Fig. 6C).

3.5. Blood metabolism mass spectrometry and data analysis

The identification, quantification, and classification results of 133 DAMs selected by non-targeted metabolomics are shown in Fig. 9. The results showed that the most abundant DAMs between DHC and DKC were mainly alkaloids and fatty acids (Fig. 7A), indicating that there

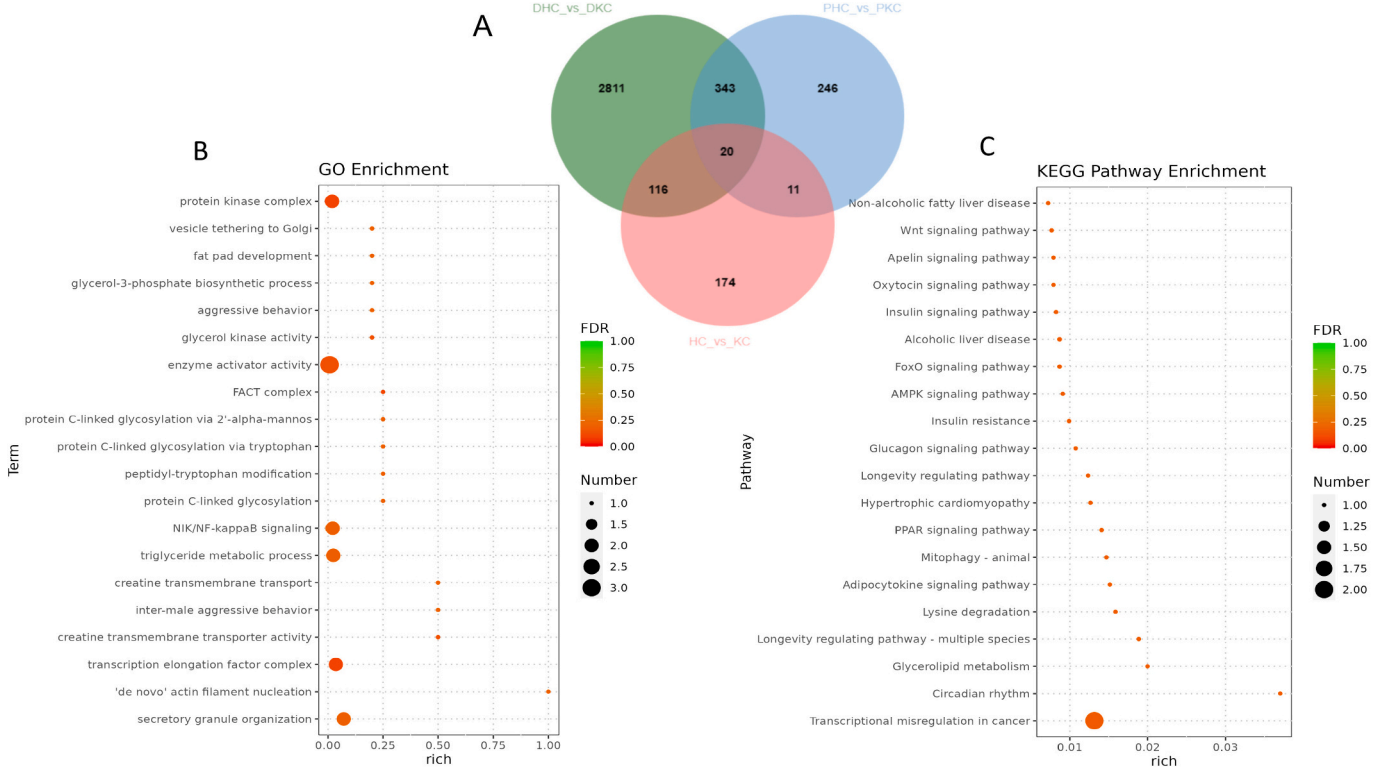

Fig. 6. GO term and KEGG-enriched pathways for DEGs shared by healthy and ketotic cows in DHC, DKC, PHC, PKC, HC, and KC.
A: Venn plot of DEGs. B: GO map of DEGs. C: KEGG pathway of shared DEGs.

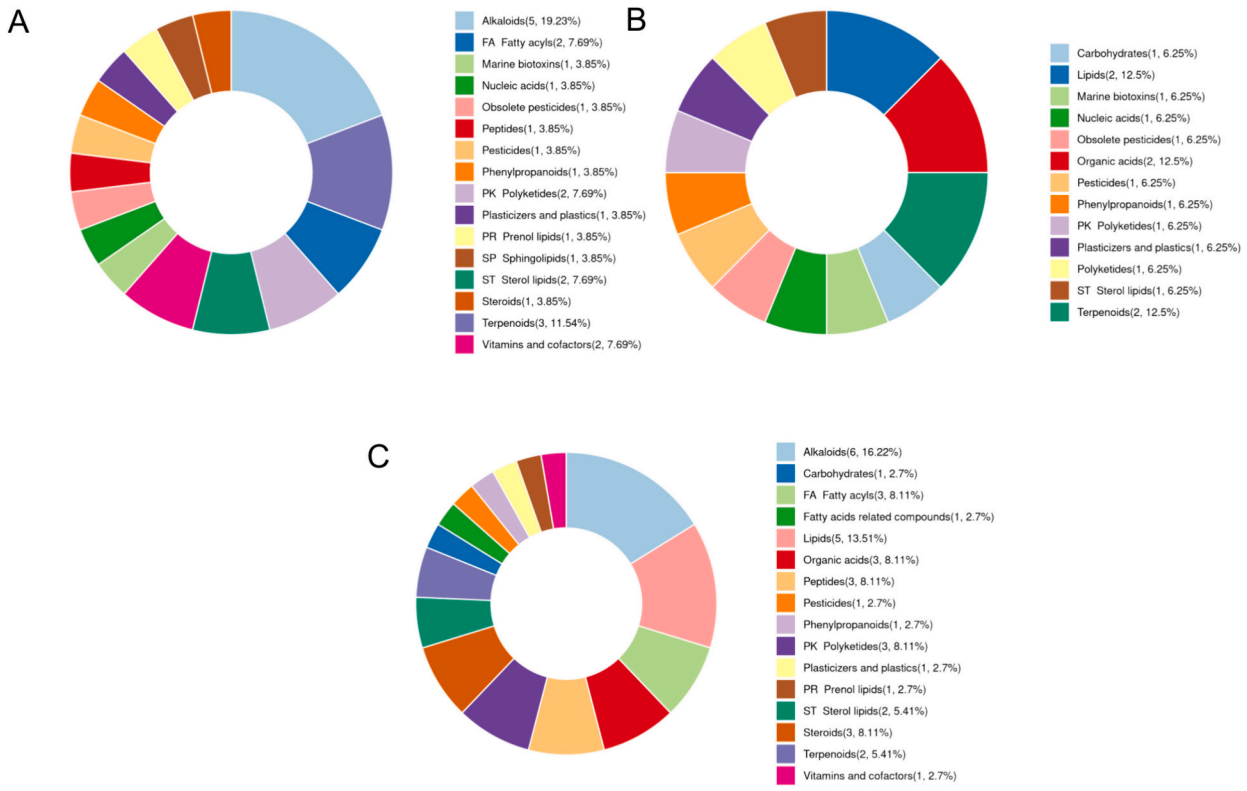

Fig. 7. Classification of DAMs in DHC, DKC, PHC, PKC, HC, and KC.
A: DHC and DKC. B: PHC and PKC. C: HC and KC.

were differences in blood alkaloids and fatty acid metabolites between DHC and DKC. The most significant DAMs between PKC and PHC were lipids (Fig. 7B), indicating that lipid metabolites differed significantly between PHC and PKC. The most significant DAMs between KC and HC were alkaloids and lipids (Fig. 7C).

Principal component analysis is an unsupervised data analysis method that examines the overall distribution trend of all samples. Analysis of all identified metabolic components in positive and negative ion modes showed that the metabolites of ketotic and healthy cows were evenly distributed during the dry period (Supplementary Fig. 5A and 5B), pre-perinatal period (Supplementary Fig. 6A and 6B), and post-perinatal period (Supplementary Fig. 8A and 8B), with significant differences in metabolites between groups.

OPLS-DA is another supervised statistical method for discriminant analysis. In this method, to predict the sample class, the relationship between metabolite expression and sample class is established by partial

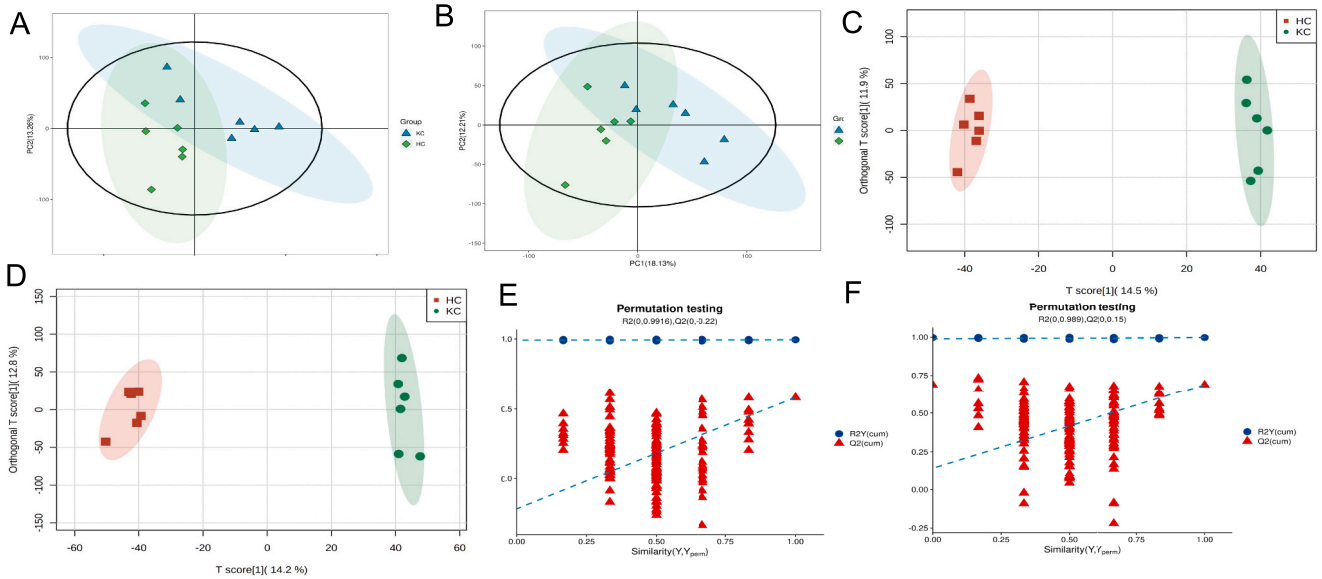

Fig. 8. PCA, OPLS-DA, and displacement tests in ketotic cows and healthy cows in post-perinatal periods.
A: PCA plot in positive ion mode. B: PCA plot in negative ion mode. C: OPLS-DA plot in positive ion mode. D: OPLS-DA plot in negative ion mode. E: Displacement test plot in positive ion mode. F: Displacement test plot in negative ion mode.

least squares regression. Generally, R2 (model fitting) and Q2 (predictive ability) are used to jointly evaluate the model, and if R2Y > 0.4 and Q2 > 0.4, the model can be considered reliable. The results of the OPLS-DA analysis of ketotic cows are detailed in Supplementary Fig. 5C and 5D, Supplementary Fig. 6C and 6D, and Fig. 8B and C. The R2Y and Q2 values of the models are both greater than 0.4, indicating that the model is reliable, and can distinguish between ketotic and healthy cows. In addition, the predicted and interpreted values of the cut-off points (R2, Q2) of ketotic and healthy cows (Supplementary Fig. 5E and 5F, Supplementary Fig. 6E and 6F, Fig. 8E and F) met the requirements, and the intercept of the regression line Q2 was less than 0. This result confirms that the OPLS-DA model was not overfitting.

Metabolites with variable importance in projection >1 and $p < 0.05$ were selected as metabolites with statistically significant differences. In this study, a total of 333 DAMs were identified between ketotic and healthy cows during dry, pre-prenatal, and perinatal stages (Table 2), 113 DAMs were identified between DKC and DHC, 85 DAMs were identified between PKC and PKC, and 135 DAMs were identified between KC and HC.

There were six DAMs among DHC, DKC, PHC, PKC, HC, and KC in the positive ion mode (Fig. 9A), which were taurolithocholic acid, purines I-IV gallate, pancuronium, di(2-ethylhexyl) phthalate, 3 beta-hydroxy-23,24-bisnorchol-5-enoic acid, and 1-desoxymethylsphinganine. There were two DAMs among DHC, DKC, PHC, PKC, HC, and KC in the negative ion mode (Fig. 9B), namely PC (18:1e/12-HETE) and methyl palmitoleate.

Functional enrichment analysis of DAMs disclosed potential metabolic changes in ketotic cows from dry milk to post-perinatal periods (Fig. 9). DAMs between DKC and DHC were mainly enriched in cofactor biosynthesis, glyoxylic acid and dicarboxylic acid metabolism, phenylalanine metabolism, lysine degradation, citric acid cycle, alanine, aspartic acid, and glutamate metabolism pathways (Fig. 9C). This enrichment pattern indicates that the occurrence and development of ketosis in cows are accompanied by changes in metabolic pathways. DAMs between PKC and PHC were mainly enriched in energy metabolism-related pathways, such as citric acid cycle and ABC transport (Fig. 9D), while the DAMs between KC and HC were mainly enriched in amino acid degradation, fatty acid metabolism, and carbon metabolism pathways (Fig. 9E). The changes of metabolic pathways above help to uncover the changes of metabolites at different physiological stages of ketotic and healthy cows.

3.6. Combined transcriptome and metabolome analysis

Combined transcriptomic and metabolomic analysis showed that there were 77 common KEGG pathways in ketotic and healthy cows, including 18 prenatal and 46 postnatal. The signaling pathways for the significant enrichment of DEGs and DEMs in ketotic and healthy cows during the dry milk stage were mainly tricarboxylic acid cycle, oxidative phosphorylation, pyruvate metabolism, and fatty acid degradation signaling pathways (Fig. 10A). Lysine degradation and fatty acid biosynthesis were the main signaling pathways of DEGs and significantly enriched DEMs between ketotic and healthy cows in the pre-perinatal period (Fig. 10B). The signaling pathways for the significant enrichment of DEGs and DAMs between ketotic and healthy cows in the post-perinatal periods were fatty acid biosynthesis, unsaturated fatty acid biosynthesis, and tricarboxylic acid cycle signaling pathways (Fig. 10C). The pathways involved in DEGs and DAMs in dry milk, pre-perinatal, and post-perinatal stages mainly include glucagon signaling pathway, lysine degradation pathway, and carbon metabolism signaling pathway in the cancer center. In addition, the results also showed that the expression levels of protein kinase amp-activated non-catalytic subunit beta 2 (*PRKAB2*) in glucagon signaling pathway and SET domain and mariner transposase fusion gene (*SETMA*R) in the lysine degradation pathway were significantly higher in the dry period, pre-perinatal period, and post-perinatal period of ketotic cows than in healthy cows.

Table 2
Shows statistical table of significant metabolites.

Sample comparison group	Significant metabolite count
DKC.vs.DHC.POS	105
DKC.vs.DHC.NEG	8
DKC.vs.DHC.Mix	113
PKC.vs.PHC.POS	53
PKC.vs.PHC.NEG	32
PKC.vs.PHC.Mix	85
KC.vs.HC.POS	87
KC.vs.HC.NEG	48
KC.vs.HC.Mix	135

4. Discussion

4.1. Biochemical index analysis

The changes of various blood parameters are powerful means to reflect the physiological and pathological state of an organism [30]. Ketosis causes impaired liver function in cows as well as measurable changes in blood parameters [13]. The cows selected for this trial were identified as ketotic cows in the post-perinatal period by blood test. The identified higher concentrations of AST, ALT, TG, BHBA, and NEFA as well as lower concentrations of GLU confirm that the cows selected for this trial were indeed ketotic. The liver is an important organ in the metabolic process of dairy cows, and a weakening liver function has a substantial impact on the level of blood parameters and the normal function of various organs in the body [10]. The degree of liver cell damage can be gauged by measuring the enzyme activity in the serum of dairy cows. AST and ALT are commonly-used indicators to evaluate normal liver function [2,18], and these are also considered to be more sensitive indicators for changes in ketotic cows [1]. In this study, significant differences in ALT activity were found between healthy cows and ketotic cows during the dry period; significant differences in ALT activity and AST activity were also found between healthy and ketotic cows during the pre-perinatal period, indicating that ketotic cows may experience a certain degree of liver damage during the dry period. Choi et al. [7] found that the TG levels associated with ketosis in the pre-perinatal period were significantly higher than the TG levels of healthy cows, indicating impaired liver lipid metabolism. In the present study, TG levels of ketotic cows in the dry period were also significantly higher than those of healthy cows. In addition, a study to prevent the occurrence of ketosis in cows showed that adding therapeutic exogenous additives to the diets of dairy cows significantly changed the levels of ALT, AST, and TG in their serum; this reduced the occurrence of ketosis, which proved that ALT and AST can indeed be used as markers to predict the occurrence of ketosis in dairy cows [12]. Therefore, the significant changes of both ALT and TG in the dry period can be used as biomarkers for the occurrence of ketosis in cows. The occurrence of ketosis in cows postpartum can be prevented and improved by identifying the differences between ALT and TG in the dry period.

Oxidative stress occurs in ketotic cows, which may be related to the higher non-esterified fatty acids (NEFA) of ketotic cows [22], while the contents of MDA, reactive oxygen species, SOD, GSH-Px, and other indexes can reflect the level of lipid peroxidation [4]. Research showed that high concentrations of NEFA and BHBA can increase the accumulation of MDA and reactive oxygen species, and reduce the activities of SOD and GSH-Px, leading to oxidative stress in dairy cows [20]. Similarly, in the present experiment, the MDA content of ketotic cows in the post-perinatal period was significantly higher, while the activities of SOD and GSH-Px were significantly lower than those of healthy cows. The MDA content of ketotic cows during the dry period was significantly higher than that of healthy cows, indicating that ketotic cows experienced oxidative stress during the dry period. In addition, high levels of NEFA in the plasma of ketotic cows activate the NF-κB pathway, and the

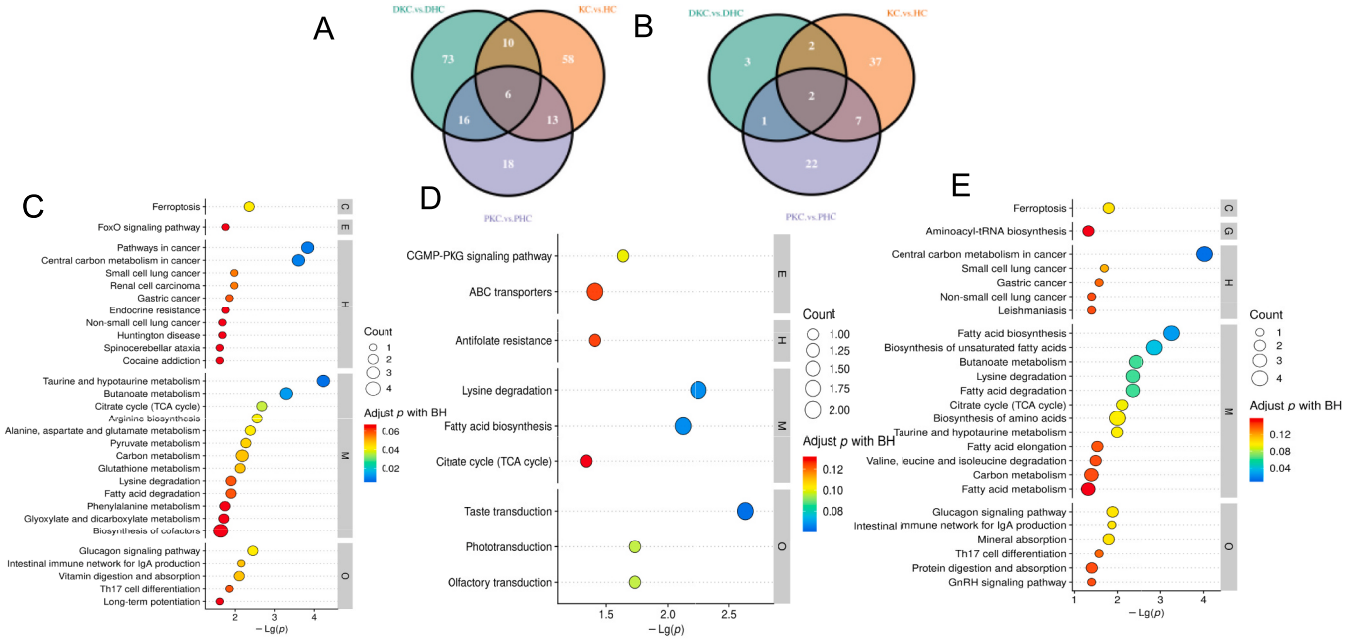

Fig. 9. Venn diagram and functional enrichment analysis of DAMs in healthy and ketotic cows in DHC, DKC, PHC, PKC, HC, and KC.
A: Venn diagram of DAMs in positive ion mode. B: Venn diagram of DAMs in negative ion mode. C: DHC and DKC. D: PHC and PKC. E: HC and KC.

activated NF-κB up-regulates the release of pro-inflammatory cytokines, leading to rapid increase in IL-6, IL-8, and TNF-α levels [35]. In this experiment, the contents of inflammatory factors IL-6, IL-8, and TNF-α increased during the post-perinatal period in ketotic cows, which confirmed this result. Serum IL-6 levels can be used as a predictive biomarker for the early onset of ketosis in dairy cows [60]. In the present experiment, the concentration of IL-6 in the blood of ketotic cows was higher than that of healthy cows during dry period, pre-perinatal periods, and post-perinatal periods. This result further confirms that IL-6 can be used as an early warning biomarker for the onset of ketosis in dairy cows.

4.2. Transcriptomics discloses important regulatory genes in the pathogenesis of ketosis in cows

RNA-Seq has been widely used in the screening and identification of genes associated with the occurrence of diseases in dairy cows. In this experiment, changes in metabolic and immune system pathways were found in ketotic dairy cows. Changes in these pathways were also found in a study that performed transcriptomics and weighted gene co-expression network analysis in ketotic cows [54]. The appearance of these changes suggests that alterations in the immune system and metabolic system may result in reduced defenses and energy utilization in cows to changes of external and internal environments, leading to severe loss of milk production and large mobilization of lipids. This also leads to greater acute phase reaction in cows after calving, which undermines their liver function. GO and KEGG analyses of DEGs in the post-perinatal periods further confirmed that ketotic cows were mainly enriched in metabolic and immune system pathways, which was consistent with the results of Shahzad et al. [34] who conducted transcriptome sequencing of the liver of ketotic cows. They also found enrichment in metabolic and immune system pathways. Interestingly, a comparison of the results with those obtained by Wu et al. [50] using transcriptome selection showed that no difference was found in STX1A in this experiment, which may be related to differences in the selected sample region. In addition, a joint analysis of the DEGs of ketotic and healthy cows during dry period, pre-perinatal period, and post-perinatal period was also conducted. Twenty shared genes were screened out that were significantly enriched in the peroxisome proliferator-activated receptor (PPAR) signaling pathway and AMPK signaling pathway, which were also identified in previous ketosis-related studies [3,21]. PPAR belongs to the family of ligand-activated nuclear receptors [43] and plays a key role in energy homeostasis, metabolism, and inflammation control [31]. PPAR forms a dimer with retinoic acid X receptor and regulates the expression of downstream target genes after ligand binding. Three subtypes of PPAR have been reported, namely PPARα, Pparβ/δ, and Pparγ [44]. PPARα, PPARβ/δ, and PPARγ all regulate lipid stabilization in the liver; PPARα regulates lipid metabolism in the liver, PPARβ/δ mainly promotes fatty acid β oxidation in the extrahepatic organs, and PPARγ stores triacylglycerol in adipocytes [47]. AMPK is a major regulator of the liver glucose metabolism [57] and activation of the AMPK signaling pathway can achieve important regulation of lipid metabolism and glucose metabolism [52]. Ketosis is often associated with disorders of glucose and lipid metabolism [33]. Activation of the AMPK signaling pathway regulates lipid metabolism in bovine hepatocytes, and the expression of PPARα and its target genes is altered [56]. Therefore, targeted regulation of both the PPAR signaling pathway and the AMPK signaling pathway may be an effective means to prevent and control the early onset of ketosis in dairy cows.

4.3. Metabolomics discloses the difference in metabolites between ketotic and healthy cows

Metabolomics is widely used for early diagnosis, biomarker screening, identification, and pathogenesis analysis of metabolic diseases. In this study, a method based on liquid chromatography-mass spectrometry non-targeted metabolomics was used to measure the plasma metabolism of healthy and ketotic cows. DAMs mainly involved amino acid metabolism, fatty acid metabolism, carbon metabolism, and fatty acid biosynthesis signaling pathways. In addition, the tricarboxylic acid cycle and metabolic pathways of alanine, aspartate, and glutamate were also significantly enriched, and these results were consistent with previous research [25]. For example, in ketotic cows, amino acid metabolic pathways, especially those related to ketogenesis were altered. The tricarboxylic acid cycle pathway plays an important role in perinatal dairy cows as the final metabolic pathway of the glycolipid

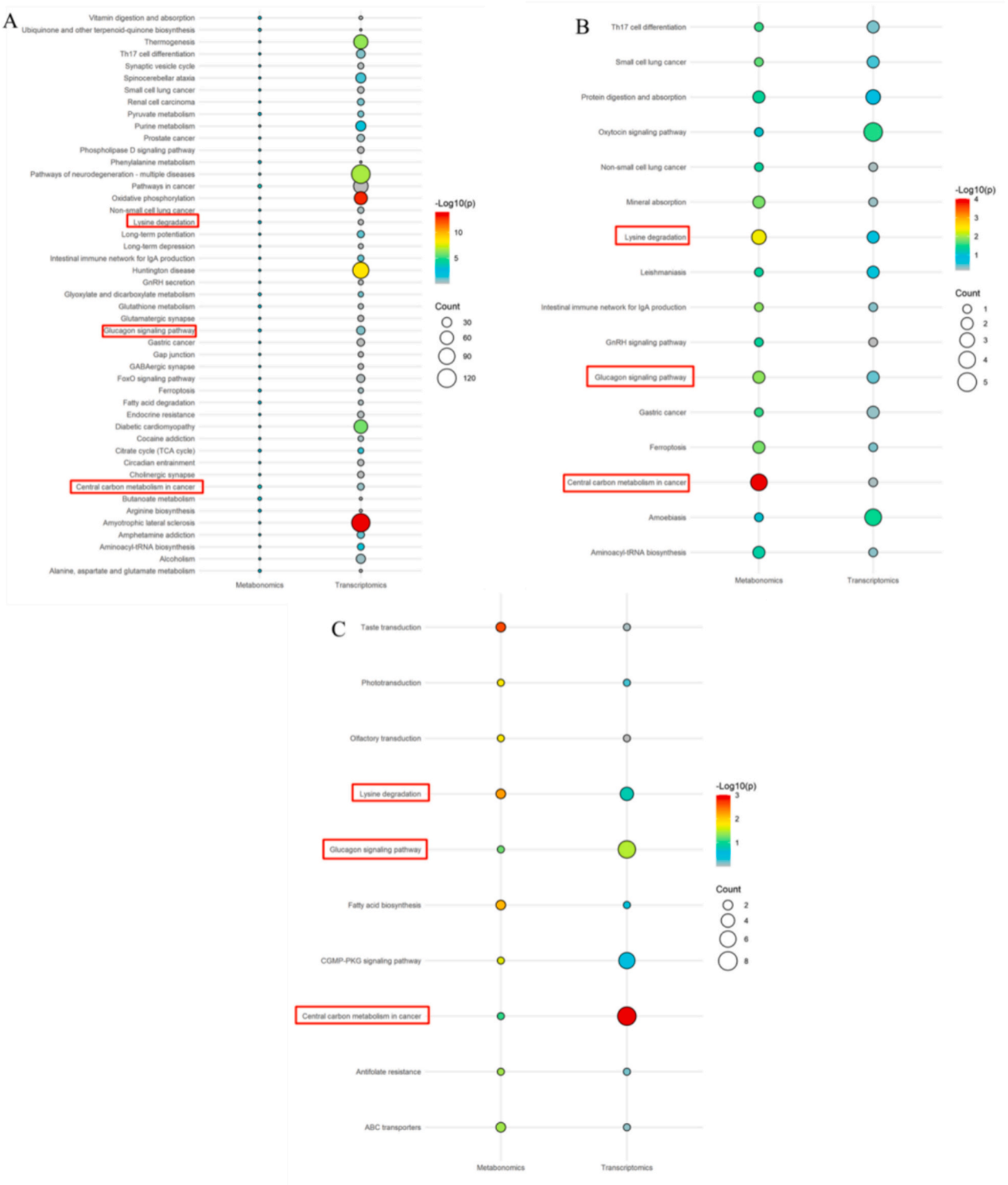

Fig. 10. Comparative bubble plots of total significant KEGG in healthy and ketotic cows in DHC, DKC, PHC, PKC, HC, and KC.
A: DHC and DKC. B: PHC and PKC. C: HC and KC.

amino acid metabolism [48]. In addition, in this study, DAMs of ketotic and healthy cows at three different stages were significantly enriched in the tricarboxylic acid cycle pathway, which has also been reported previously [49]. Therefore, the results of this experiment indicate that ketotic cows may show differences in energy metabolism during the dry period.

In addition, a comparison of differential metabolite enrichment pathways among healthy and ketotic cows during dry, pre-perinatal, and post-perinatal periods showed that the enriched pathways include butyric acid metabolism, carbon metabolism, lysine degradation, and fatty acid degradation. Most of these pathways play important roles in the occurrence and development of ketosis [34,38,46]. Studying these pathways can provide the basis for the future in-depth disclosure of the mechanism of the occurrence of ketosis as well as a new strategy for its prevention and treatment. In addition, amino acids, glycerophospholipids, sphingolipids, acylcarnitine, bioamines, and

methylglyoxal have been predicted and identified as biomarkers of the ketosis metabolism in dairy cows [19,59]. Interestingly, the common DAMs of ketotic cows and healthy cows mainly included taurolithocholic acid and methyl palmitoleate during the dry, pre-perinatal, and post-perinatal periods. In conclusion, in addition to amino acids, glycerophospholipids, sphingolipids, acylcarnitine, bioamines, and methylglyoxal, taurolithocholic acid and methyl palmitate can also be used as early metabolic biomarkers of ketosis in cows.

4.4. Combined multi-omics analysis discloses key pathways and genes for the development of ketosis in dairy cows

Combined transcriptome and metabolome analysis showed that glucagon signaling pathway, lysine degradation signaling pathway, and cancer center carbon metabolism signaling pathway play important regulatory roles in the occurrence of ketosis in cows. Mining of jointly involved genes in these signaling pathways showed that PRKAB2 and SETMAR were upregulated in both DKC and perinatal ketotic cows, suggesting that these two genes may be key genes in the development of ketosis in dairy cows. PRKAB2 belongs to the protein kinase family and plays a key role in the regulation of the energy metabolism in eukaryotic cells [16]. Activated PRKAB2 initiates the ATP-producing pathway and inhibits the consumption of ATP [42]. PRKAB2 plays an important role in the lipid and glucose metabolism by blocking the phosphorylation of glycogen synthase and stimulating fatty acid oxidation [14,53]. However, few studies have focused on PRKAB2, with research in pigs confirming that PRKAB2 affects meat quality traits [55] and research in chickens confirming that PRKAB2 affects slaughtering traits and meat quality traits [45]. The mechanism of PRKAB2 in the pathogenesis of ketosis in cows needs to be further determined in the future. SETMAR is a protein lysine methyltransferase involved in DNA processes such as DNA repair in non-homologous end-joining pathways, gene expression regulation, and DNA deamidation [40]. Most of the current studies on SETMAR focused on its function in different types of cancer [6,41,51], while studies on SETMAR in livestock and poultry are rare. In this study, SETMAR was upregulated in both dry and perinatal ketotic cows, and can be used as a diagnostic gene for the early onset of ketosis in cows. However, its specific criteria and mechanism need to be further studied. In addition, joint analysis found the DAM acetoacetate in the blood of cows in both the pre-perinatal and post-perinatal periods, which may also be a marker of the incidence of ketosis in cows during the perinatal period.

5. Conclusion

Protein content analysis identified ALT, TG, and IL-6 as biomarkers predicting the development of ketosis during the dry milking period. Transcriptomic and metabolomic results illustrated DEGs and mainly genes such as BRWD1, REL, and ENSBTAG00000050908 as well as signaling pathways such as tricarboxylic acid cycle, butyric acid metabolism, carbon metabolism, lysine degradation, and fatty acid degradation in cows with ketosis from the dry milk stage to the periparturient stage. The combination of transcriptomics and metabolomics indicated that glucagon and lysine degradation pathways may be key signaling pathways for the development of ketosis in dairy cows, and PRKAB2 and SETMAR may be candidate genes for the early onset of ketosis. This study provides a new method for the detection and diagnosis of early ketosis in dairy cows, which enables early detection and treatment to improve the performance and health of dairy cows.

Institutional review board statement

This experimental procedure was approved by the Animal Experimentation Committee of Ningxia University in accordance with the Regulations on the Management of Laboratory Animals in China (Ningxia University Ethics No. 22–72). The experimental procedures were performed in strict accordance with the approved guidelines and regulations.

CRediT authorship contribution statement

Sha Ping: Writing – original draft, Formal analysis, Conceptualization. **Ma Xuehu:** Writing – original draft, Formal analysis, Conceptualization. **Hu Chunli:** Software, Data curation. **Feng Xue:** Visualization, Validation. **An Yanhao:** Visualization, Validation. **Ma Yanfen:** Visualization, Validation.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that could unduly influence our work, and that we have no professional or other personal interests of any nature or type with any product, service and/or company that could be perceived to influence the content of this article.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2024.110927.

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Glossary of abbreviated words

Abridge: Full name
NEB: negative energy balance
BHBA: beta-hydroxybutyric acid
DHC: dry period healthy cows
DKC: dry period ketosis cows
PHC: pre-perinatal healthy cows
PKC: pre-perinatal ketosis cows
HC: post-perinatal healthy cows
KC: post-perinatal ketosis cows
DEGs: differentially expressed genes
DAMs: differentially accumulated metabolites
ALT: alanine aminotransferase
AST: glutamine aminotransferase
TG: triglyceride
GLU: glucose

GSH-Px: glutathione peroxidase
MDA: malondialdehyde
SOD: superoxide dismutase
IL-6: interleukin-6
IL-8: interleukin-8
TNF-α: tumor necrosis factor
PRKAB2: protein kinase amp-activated non-catalytic subunit beta 2
SETMAR: SET domain and mariner transposase fusion gene
OPLS-DA: orthogonal partial least squares discriminant analysis
PPAR: proliferator-activated receptor