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Original Research Article

Metabolomic and transcriptomic study to understand changes in metabolic and immune responses in steers under heat stress

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ABSTRACT

Heat stress (HS) damages livestock by adversely affecting physiological and immunological functions. However, fundamental understanding of the metabolic and immunological mechanisms in animals under HS remains elusive, particularly in steers. To understand the changes on metabolic and immune responses in steers under HS condition, we performed RNA-sequencing and proton nuclear magnetic resonance spectroscopy-based metabolomics on HS-free (THI value: 64.92 ± 0.56) and HS-exposed (THI value: 79.13 ± 0.56) Jersey steer (n = 8, body weight: 559.67 ± 32.72 kg). This study clarifies the metabolic changes in 3 biofluids (rumen fluid, serum, and urine) and the immune responses observed in the peripheral blood mononuclear cells of HS-exposed steers. This integrated approach allowed the discovery of HS-sensitive metabolic and immunological pathways. The metabolomic analysis indicated that HS-exposed steers showed potential HS biomarkers such as isocitrate, formate, creatine, and riboflavin ($P < 0.05$). Among them, there were several integrative metabolic pathways between rumen fluid and serum. Furthermore, HS altered mRNA expression and immune-related signaling pathways. A meta-analysis revealed that HS decreased riboflavin metabolism and the expression of glyoxylate and dicarboxylate metabolism-related genes. Moreover, metabolic pathways, such as the hypoxia-inducible factor-1 signaling pathway, were downregulated in immune cells by HS ($P < 0.05$). These findings, along with the datasets of pathways and phenotypic differences as potential biomarkers in steers, can support more in-depth research to elucidate the inter-related metabolic and immunological pathways. This would help suggest new strategies to ameliorate the effects of HS, including disease susceptibility and metabolic disorders, in Jersey steers.

1. Introduction

Although livestock have evolved diverse physiological mechanisms to cope with environmental changes, there are numerous challenging events that may evoke a stress response. Hot and humid environments have been considered hazardous for diverse physiological aspects, including metabolism and immunity, in livestock. Since heat stress (HS) imposes significant economic burden on livestock rearing operations (such as dairy cows, steers

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and chickens), and thus active mitigation efforts (such as employing diverse nutritional strategies and introducing cooling systems in barns) are being carried out (St-Pierre et al., 2003; Min et al., 2019). However, global warming is a major concern for the animal industry worldwide. The most prominent negative impact of HS on animals is reduced growth performance (Broucek et al., 2009; He et al., 2020; Siddiqui et al., 2020). In most cases, reduced growth performance in animals is explained by adverse changes in metabolism, induced by HS (Drackley et al., 2005; Pearce et al., 2013; Gao et al., 2017; Lu et al., 2018). HS-exposed animals also suffer frequently from metabolic diseases induced by abnormal levels of certain metabolites (Russell and Rychlik, 2001; Gantner et al., 2016), and therefore, some metabolites can potentially be used as biomarkers to diagnose metabolic diseases and to understand their pathological aspect (Zhang et al., 2019). For example, presence of non-esterified fatty acids (NEFA) and aspartate aminotransferase (AST) in blood during the early lactation period is a sign of mastitis in dairy cows (Moyes et al., 2009). Related studies also suggest that decreased levels of plasma β-hydroxybutyrate (BHBA) and citrate in dairy cows may be used as biomarkers to evaluate their susceptibility to HS and the energy balance, respectively (Dhillon and Singh, 2013; Yue et al., 2020). However, there is limited information on changes in metabolites during HS and the mechanism by which HS response induces metabolic diseases. This makes it difficult to discover useful biomarkers for diagnosing preclinical metabolic diseases in livestock animals. Therefore, metabolomics is often used to obtain comprehensive metabolic information and to explore complex metabolic pathways (Yang et al., 2018). Metabolomics also plays a pivotal role in systemic biology by diagnosing diseases, and discovering biomarkers and novel metabolic pathways in clinical studies (Johnson et al., 2016). Metabolomic studies using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy have been performed to investigate the inherent metabolic patterns under HS by analyzing various biofluids, including rumen fluid, serum, plasma, milk, urine, and feces of ruminants (Min et al., 2017; Yue et al., 2020).

Immunological responses are also altered by HS and may be associated with susceptibility to infectious and non-infectious diseases in livestock. A correlation study reported that heat-stressed animals displayed increased incidence of infectious and metabolism-related disease (Collier et al., 1982; Wheelock et al., 2010). Improper regulation of host metabolism increases the vulnerability to metabolic disease. In addition, HS-mediated physiological changes can induce abnormal immune responses in animals through diverse mechanisms. Several studies have also shown that Holstein bull calves under HS show hyperinsulinemia, which leads to changes in expression of genes related to immune response (Srikanth et al., 2017). In addition, the expression of interferon gamma (IFN-γ) in $CD4^+$ and $CD8^+$ lymphocytes was also suppressed under HS, which may explain the increased susceptibility of animals to pathogenic infections and infectious diseases (Hu et al., 2007; Park et al., 2021). Relevant studies have suggested that heat-stressed animals may have improper immune function. For instance, suppression of cytokine expression in T lymphocytes and a shift in immune response from T helper 1 (Th1) to T helper 2 (Th2) cells in bull cattle under HS reflected depression of cell-mediated immunity (Hu et al., 2007; Peli et al., 2013). In addition, our previous study revealed that HS induced altered immunity by changing the immune cell population and their functions, which could lead to the deterioration of the immune homeostasis in steers (Park et al., 2021). However, functional changes in the immunity of steers induced by HS have not been fully investigated. RNA-sequencing (RNA-seq) is one of the most biologically relevant tools to investigate functional changes occurring in host tissue under specific experimental conditions, such as HS, through analyzing mRNA expression (Costa et al., 2013). Considering transcriptional regulation, which plays a major role in eliciting appropriate immune responses, RNA-seq analysis provides key information on the development of immunological profiles of animals based on specific gene expression related to immune cells.

The objective of this study was to understand the changes in metabolic and immunological responses in Jersey steers under HS. Jersey steers are an important source of meat worldwide as they have superior tenderness compared to other breeds (Purchas and Barton, 1976). However, since the market for beef cattle is smaller than that for dairy cows, research on physiological and immunological aspects of steers has not been actively conducted. Thus, a correlated study to understand the physiological and immunological characteristics of Jersey steers as well as understanding the modulation of these characteristics by HS is essential for the beef industry. A metabolomic study using ^{1}H-NMR spectroscopy for 3 biofluids (rumen fluid, serum and urine), and immunological studies using RNA-seq for peripheral blood mononuclear cells (PBMCs) were performed to understand the differential biological responses with a focus on metabolism and immune responses in Jersey steers under a moderate temperature period (MTP) and a high temperature period (HTP) conditions. This integrative analysis allows us to understand complex physiological and immunological responses and their potential interactions in heat-stressed animals. Moreover, this would help to develop new strategies, including the discovery of diagnostic biomarkers for diseases and dietary intervention, to ameliorate the adverse effects of HS.

2. Materials and methods

2.1. Animal ethic statement

All experimental procedures for animal experiments were performed in accordance with the guidelines (SCNU-IACUC-2020-06) of the Institutional Animal Care and Use Committee (SCNU-IACUC), Republic of Korea.

2.2. Animals and sample collections

Eight Jersey steers (body weight: 559.67 ± 32.72 kg) were maintained under two separate 3 × 3 Latin square design areas. All steers were kept in individual stalls with feeding and water facilities, and fed only basal total mixed ration (TMR) once a day at 09:00, with a feed refusal rate of 5% to 10%. The chemical composition of the feed is presented in Table 1. The contents of dry matter (Method 934.01), crude protein (Method 976.05), crude fat (Method 920.39), ash (Method 942.05), calcium (Method 927.02), and phosphorus (Method 3964.06) in TMR were assayed as described by the Association of Official Analytical Communities methods (AOAC, 2003; AOAC, 2005). The contents of neutral detergent fiber and acid detergent fiber in the TMR were assayed as described by Van Soest et al. (1991). The biofluid samples were collected under different temperature humidity index (THI). With respect to livestock, THI is mainly used to reflect the intensity of HS. Accordingly, based on the available meteorological data, we used the THI to examine the effects of the HS on Jersey steers. To measure the degree of HS in steers, two time slots according to the THI were assessed for comparative studies. For the determination of THI, we measured temperature and humidity using a temperature and humidity meter (Testo 174H Mini data logger; West Chester, PA, USA) and applied the following THI equation, devised by the National Research Council (NRC, 1971):

Table 1
Ingredients and chemical composition of total mixed ration (DM basis, %).

Item	Content
Ingredients	
Corn gluten meal	8.40
Soybean	6.24
Beet pulp	4.20
Wheat bran	3.15
Corn flakes	2.21
Molasses	1.04
Rice wine residue	5.25
Brewer's grain residue	21.01
Annual ryegrass straw	27.29
Orchard grass straw	21.01
Limestone	0.10
Sodium bicarbonate	0.01
Salt	0.09
Total	100.00
Chemical composition	
DM (fresh basis)	55.32
Crude protein	13.51
Crude fiber	22.12
Crude fat	3.16
Ash	9.22
Calcium	1.54
Phosphorus	0.58
Neutral detergent fiber	49.12
Acid detergent fiber	25.80

$$\begin{aligned}\mathrm{THI} = {} & (1.8 \times \text{ambient temperature} + 32) \\ & - [(0.55 - 0.0055 \times \text{relative humidity}) \\ & \times (1.8 \times \text{ambient temperature} - 26)]\end{aligned}$$

To calculate the THI, measurements were conducted for MTP from May 14th to 27th and for HTP from August 5th to 13th, for a total of 18 d. As a result, we obtained average THI values of 64.92 ± 0.56 (mean ± standard error of the mean [SEM]) and 79.13 ± 0.56 (mean ± SEM), respectively. Rumen fluid, serum and urine samples were collected before the morning feeding. Rumen fluid was collected from Jersey steers using a stomach tube ($n = 8$/group) (Shen et al., 2012). The first rumen fluid was not sampled because of saliva and blood contamination. The samples were centrifuged at 806 × *g* at 4 °C for 15 min to remove feed particles, and the supernatant was stored at −80 °C for ^{1}H-NMR spectroscopy analysis. Blood from the jugular neck vein was collected in a serum-separating tube (BD Vacutainer, SST II advance, Becton Dickinson Co., Franklin Lakes, NJ, USA) from Jersey steers ($n = 8$/group). The blood samples were centrifuged at 806 × *g* at 4 °C for 15 min, and aliquots of the upper layer (serum) were stored at −80 °C for ^{1}H-NMR spectroscopy analysis. Urine samples were collected in freshly cleaned cups and immediately stored at −80 °C for ^{1}H-NMR spectroscopy analysis (MTP, $n = 4$; HTP, $n = 8$).

2.3. Sample preparation for ^{1}H-NMR spectroscopy

The rumen fluid sample was centrifuged at 12,902 × *g* at 4 °C for 10 min, and 300 μL of the supernatant was collected. A standard buffer solution (2,2,3,3-d(4)-3-(trimethylsilyl) propionic acid [TSP] sodium salt) was added to 300 μL of the supernatant in deuterium oxide (D_2O) solvent/standard buffer solution (300 μL). The supernatants (600 μL) were transferred to 5 mm NMR tubes for ^{1}H-NMR spectroscopy analysis (O'Callaghan et al., 2018). We prepared saline buffer (concentration of 0.9% wt/vol) by applying NaCl in 100% D_2O solvent. The stored serum samples were centrifuged at 14,000 × *g* at 4 °C for 10 min. The supernatant (200 μL) was added to 400 μL of saline buffer in 5 mm NMR tube for ^{1}H-NMR spectroscopy analysis (Sun et al., 2014). The urine samples were centrifuged at 14,000 × *g* at 4 °C for 10 min, and 400 μL supernatant was collected. Supernatant (400 μL) was added to 230 μL 0.2 M sodium phosphate buffer and was measured of pH 7.0 ± 0.1. The mixture solution (630 μL) was added to 2 mM TSP (60 μL) to adjust the final TSP concentration to 0.2 mM (Bertram et al., 2011). The prepared sample was transferred to a 5 mm NMR tube for ^{1}H-NMR spectroscopy analysis.

2.4. ^{1}H-NMR spectroscopy

The spectra of rumen fluid, serum and urine samples were obtained on a SPE-800 MHz NMR-MS Spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) equipped with a 5 mm triple-resonance inverse cryoprobe with Z-gradients (Bruker BioSpin Co., Billerica, Massachusetts, USA). The pulse sequence used for the two samples (rumen fluid and serum) and urine were a Carr-Purcell–Meiboom–Gill pulse sequence and NOESY presaturation, respectively. We collected 64,000 data points with 128 transients, a spectral width of 16,025.641 Hz, a relaxation delay of 4.0 s, and an acquisition time of 2.0 s (Kim et al., 2019).

2.5. Metabolites identification, quantification, and statistical analyses

Metabolite identification and quantification were carried out by importing the analyzed spectral data into the Chenomx NMR suite 8.4 software (ChenomxInc, Edmonton, Alberta, Canada). The baseline and phase were matched for comparison between samples using the Chenomx processor. The spectral width was δ 0.2 to 10.0 mg/kg and was referenced to the TSP signal at 0.0 mg/kg. Qualitative and quantitative metabolite analyses were performed using the Livestock Metabolome Database (http://www.lmdb.ca), Bovine Metabolome Database (http://www.bmdb.ca) and the Chenomx profiler. Statistical analyses of the metabolite data were performed using Metaboanalyst (version 5.0; http://www.metaboanalyst.ca). To perform a standard cross-sectional two-periods study, we compared the periods of MTP and HTP conditions. The resulting data were processed by normalization-selected methods, followed by sample normalization via normalization to constant sum, data transformation via log normalization and data scaling via pareto scaling (Torgrip et al., 2008). The rumen fluid, serum and urine metabolite data with 50% of samples under the identification limit or with at least 50% of values missing were eliminated from the analysis. The metabolites that were missing values were replaced by a value of one-half of the minimum positive in the original data. The univariate Student's *t*-test was used to quantify differences between in the metabolite profiles of the rumen fluid, serum and urine under the MTP and HTP conditions. Principal components analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were used as multivariate data analysis techniques to generate a classification model and provide quantitative information for discriminating rumen fluid, serum and urine metabolites. The different groups of rumen fluid, serum and urine metabolites from MTP and HTP conditions were determined based on a statistically significant threshold of variable importance in projection (VIP) scores. Metabolites with VIP scores higher than 1.5 were obtained using the PLS-DA model. Metabolic pathway analysis was performed using the *Bos taurus* pathway library (Kyoto Encyclopedia of Genes and Genomes [KEGG], http://www.kegg.com). Significantly different metabolic pathways in the rumen fluid, serum and urine metabolites of the other study animals were statistically analyzed and determined using Metaboanalyst 5.0, which is based on the database source by KEGG.

2.6. Blood collection and immune cell isolation

Jersey steer whole blood was obtained from the jugular vein into Vacutainer tubes (BD Vacutainer, Becton Dickinson Co., Franklin Lakes, NJ, USA); a plastic whole blood tube spray-coated with K_2EDTA. For isolation of peripheral blood mononuclear cells (PBMCs), whole-blood samples were diluted with phosphate buffered saline (PBS) to a 1:1 ratio in 15 mL conical tubes. Next, the diluted blood samples were overlaid on top of Lymphoprep (STEMCELL Technologies Inc., Vancouver, BC, Canada). After centrifugation for 20 min at 800 × *g* at room temperature without breaking, the layer of cells above the Lymphoprep were collected and washed twice with PBS to obtain the purified dairy cow PBMCs. The purified PBMCs were suspended in 1 mL of Trizol reagent (Invitrogen, CA, USA) and transferred into a 1.5 mL tube. PBMCs were immediately stored at −80 °C until RNA isolation.

2.7. RNA-seq data and statistical analysis

RNA samples with an RNA integrity value of 7.0 or higher were used in RNA library construction. The mRNA-seq sample was obtained using the Illumina TruSeq Standard RNA Sample Preparation Kit (Illumina, Inc., San Diego, CA, USA). Briefly, rRNA was removed from total RNA and the remaining mRNA was fragmented into small pieces, followed by thermal mRNA fragmentation. The cleaved mRNA fragments were transcribed into first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using reverse transcriptase and random primers. Next, cDNA was synthesized to second-strand cDNA using DNA Polymerase I, RNase H, and dUTP. After the end repair process, the addition of a single 'A' bases to the fragments and adapters were ligated. Finally, the products were purified and enriched by PCR to create the final cDNA libraries (Macrogen, Seoul, Korea). RNA-seq included analysis of quality control, abundance estimation, and differential expression. Differentially expressed genes (DEGs) were statistically analyzed for functional annotation and pathway analysis. A KEGG pathway analysis was also performed on DEGs, which enabled determination of the biological pathway analysis most relevant to the DEGs. The significance of the pathways was determined using the modified Fisher's exact test ($P < 0.05$). The *P*-values were corrected using the false discovery rate (FDR) method.

3. Results

3.1. Classification of metabolites identified in 3 biofluids from Jersey steers sampled during MTP and HTP conditions

The metabolites detected and through ^{1}H-NMR spectroscopy in the 3 biofluids (rumen fluid, serum and urine) from Jersey steers are described in Fig. 1A to C. The number of metabolites detected in rumen fluid samples during MTP and HTP conditions are described in Fig. 1A. In the MTP condition, 202 metabolites were detected and 62 were quantified. In the HTP condition, 135 metabolites were detected and 38 metabolites were quantified. The number of metabolites detected in serum samples during MTP and HTP conditions are described in Fig. 1B. In the MTP condition, 159 metabolites were detected and 52 were quantified. In the HTP condition, 172 metabolites were detected and 39 metabolites were quantified. The number of metabolites detected in urine samples during MTP and HTP conditions are described in Fig. 1C. In the MTP condition, 204 metabolites were detected and 114 were quantified. In the HTP condition, 224 metabolites were detected and 109 were quantified. The commonality of the quantified metabolites in 3 biofluids in MTP and HTP conditions are presented in Fig. 1D. In the MTP condition, 20 metabolites were common between the rumen fluid and serum, 41 were common between the rumen fluid and the urine, and 36 were common between the serum and the urine. In addition, 15 metabolites were common in all the three biofluids. In the HTP condition, 14 metabolites were common between the rumen fluid and the serum, 20 were common between the rumen fluid and the urine and 28 were common between the serum and the urine. In addition, 8 quantified metabolites were found to be common in all the 3 biofluids. Assessed collectively, the number of metabolites detected and quantified in the 3 biofluids of Jersey steers decreased during HTP.

3.2. Multivariate statistical analysis of metabolite profiles of the 3 biofluids from Jersey steers sampled during MTP and HTP conditions

Based on the data obtained from ^{1}H-NMR spectroscopy, PCA and PLS-DA of the rumen fluid, serum and urine samples were performed to interpret and obtain metabolic profiles (Fig. 2). We observed highly clustered samples in rumen fluid and urine collected during HTP, distinct from that observed in biofluids collected during MTP. The PCA score plots from rumen fluid showed the most dramatic changes in the metabolic profile (PC 1: 18% and PC 2: 12.2%) (Fig. 2A). In contrast, the metabolites from the serum (PC 1: 16.9% and PC 2: 12.6%) and the urine (PC 1: 22.8% and PC 2: 13.6%) did not show a clear distinction (Fig. 2A). In the PLS-DA score plots, the samples were clearly demarcated into two parts as the temperature changed from MTP to HTP conditions, which refers to changes in the metabolic profiles of the 3 biofluid samples (rumen fluid, component 1: 17.5% and component 2: 9.5%; serum, component 1: 16.2% and component 2: 7.6%; urine, component 1: 19.9% and component 2: 12.1%) (Fig. 2B). Interestingly, as shown in the PLS-DA score plots, we observed less variation in the metabolites from the rumen fluid and urine samples obtained during HTP, compared with that observed in samples obtained during MTP (Fig. 2B).

3.3. Differentially expressed metabolites in 3 biofluids from Jersey steers sampled during MTP and HTP conditions

The differentially expressed metabolites in the 3 biofluid samples were investigated, based on their relative intensities during the MTP and the HTP conditions. The metabolomic profiles from the 3 biofluids were generally clustered together in MTP or in HTP conditions (Fig. 3A and Tables S1 to S3). In the rumen fluid samples; riboflavin, isocitrate, formate, lactose, and propionate were significantly ($P < 0.05$) higher in the MTP than in the HTP. In contrast, methanol, butyrate, betaine, 3,4-dihydroxybenzeneacetate, and homogentisate were significantly ($P < 0.05$) higher in the HTP than in the MTP. In the serum samples; methanol, ribose, ascorbate, glucitol, and levulinate were significantly ($P < 0.05$) higher in the MTP than in the HTP. In contrast, formate, glycine, anserine, lactate, and creatine were significantly ($P < 0.05$) higher in the HTP than in the MTP. In the urine samples; choline, creatinine, imidazole, trimethylamine *N*-oxide, and melatonin were significantly ($P < 0.05$) higher in the MTP than in the HTP. In contrast, 2-hydroxy-3-methylvalerate, allantoin, threonate, gluconate, and glucose were significantly ($P < 0.05$) higher in the HTP than in the MTP.

As shown in Fig. 3B, it represents the top 20 metabolites in rumen fluid, serum and urine samples with VIP score >1.5, as determined by PLS-DA model. In the rumen fluid samples, lactose (VIP score: 2.69), caffeine (2.57), isocitrate (2.52), glycylproline (2.42), and riboflavin (2.39) had the highest VIP scores in the MTP compared to those in the HTP. In contrast, homogentisate (1.93), betaine (1.75), 3,4-dihydroxybenzeneacetate (1.74), trans-aconitate (1.66), and methanol (1.61) had the highest VIP scores in the HTP compared to those in the MTP. In the serum samples; ribose (2.66),

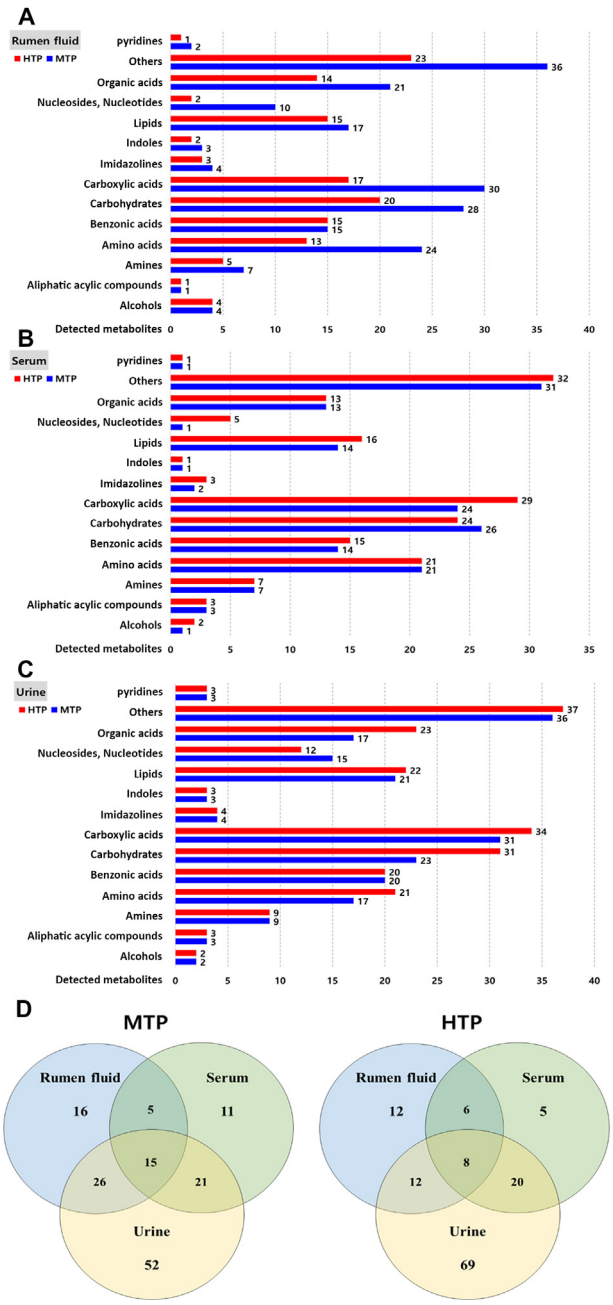

Fig. 1. Identified and quantified metabolites identified in the rumen fluid, serum and urine from Jersey steers under moderate temperature period (MTP) and high temperature period (HTP) conditions. (A to C) Detected metabolites in each category in the rumen fluid (n = 8/group under both conditions), serum (n = 8/group under both conditions) and urine (n = 4/group under MTP condition, n = 8/group under HTP condition). (D) The numbers of unique and common quantified metabolites under MTP and HTP conditions are described.

methanol (2.59), glucitol (2.28), ascorbate (2.05), and galactitol (1.73) had the highest VIP scores in the MTP compared with those in the HTP. In contrast, 2-hydroxyphenylacetate (2.41), glycolate (2.02), 5-hydroxyindole-3-acetate (1.88), acetoin (1.84), and galactonate (1.84) had the highest VIP scores in the HTP compared to those in the MTP. In the urine samples; choline (2.39), melatonin (2.01), trimethylamine *N*-oxide (1.87), and syringate (1.68) had the highest VIP scores in the MTP compared with those in the HTP. In contrast, threonate (2.71), 2-hydroxy-3-methylvalerate (2.44), gluconate (2.26), niacinamide (2.16), and 3-hydroxyisobutyrate (2.10) had the highest VIP scores in the HTP compared with those in the MTP. In this analysis, the differential metabolites identified could serve as potential biomarkers for determining HS in Jersey steers.

3.4. Metabolic pathway analysis for metabolomes in 3 biofluids under different THI

The metabolome view map was analyzed to characterize significantly different metabolic pathways observed in the 3 biofluids under changing THI condition (Fig. 4 and Tables S4 to S6). Twelve pathways showed significance in both rumen fluid and serum, while in the comprehensive results, 9 pathways were identified in urine. There were 4 pathways that had an impact value higher than 0.1, which is the cutoff value for relevance in the rumen fluid, including riboflavin metabolism (impact value: 0.50), histidine metabolism (0.34), starch and sucrose metabolism (0.19), and pyrimidine metabolism (0.13) (Fig. 4A and Table S4). In the serum, there were 3 pathways observed with impact values higher than 0.1, including synthesis and degradation of ketone bodies (0.60), glyoxylate and dicarboxylate metabolism (0.19), and butanoate metabolism (0.11) (Fig. 4B and Table S5). In addition, there were 3 pathways observed in the urine that had an impact value over 0.1, which are glutathione metabolism (0.26), nicotinate and nicotinamide metabolism (0.19), and cysteine and methionine metabolism (0.14) (Fig. 4C and Table S6). The results of the enrichment and impact pathways of the 3 biofluids demonstrated that there were several common metabolic pathways in numerous combinations of the 3 biofluids. For example, glyoxylate–dicarboxylate metabolism and tyrosine metabolism have been identified as common pathways between rumen fluid and serum. In addition, two common pathways, including glutathione metabolism and glycine–serine–threonine metabolism, were identified for rumen fluid and urine. Lastly, tryptophan metabolism and valine–leucine–isoleucine degradation were identified as common pathways between serum and urine (Tables S4 to S6). However, there was no common pathway to all 3 biofluids.

The Fig. 5 shows the integrated overview of the metabolic pathways of the rumen fluid and serum, with significantly different metabolites under MTP and HTP conditions. There were eight unique metabolome pathways from rumen fluid and serum, showing the interactions among the 12 metabolic pathways that were significantly different under HS. The metabolic pathways are all related. First, in the rumen fluid, β-alanine metabolism, pyrimidine metabolism, and riboflavin metabolism are interconnected. In addition, histidine, uracil, and riboflavin were observed to decrease in each metabolic pathway under HTP condition. Additionally, there was an interaction between rumen fluid tyrosine metabolism and the citrate cycle. Furthermore, serum showed a major correlation between pyruvate metabolism and glyoxylate and dicarboxylate metabolism. These metabolic pathways are also involved in the synthesis and degradation of ketone bodies and in tyrosine metabolism. Additionally, the pentose phosphate pathway, galactose metabolism, and ascorbate and aldarate metabolism were interconnected in the serum. More importantly, there were interactions between rumen fluid metabolites and serum metabolites. For example, the decreased rumen fluid riboflavin in riboflavin metabolism was associated with decreased ribose in the pentose phosphate pathway in serum through D-ribose-5P. In addition, increased betaine in glycine, serine, and threonine metabolism was associated with increased formate and lactate in pyruvate metabolism through pyruvate. Collectively, specific rumen fluid metabolites may affect the regulation of metabolites in serum and ultimately induce the adjustment of metabolic pathways during HTP.

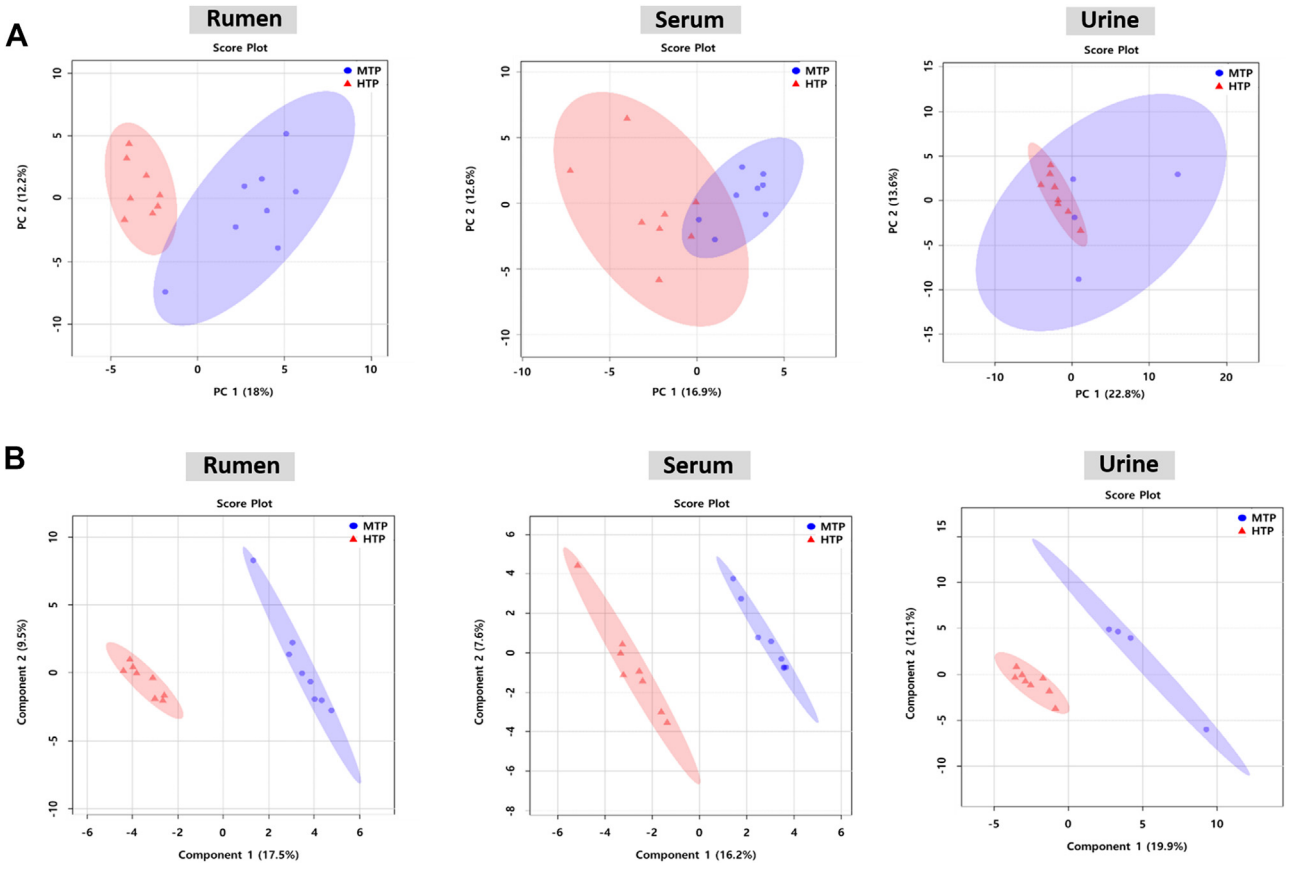

Fig. 2. Principal components analysis (PCA) score plot and partial least square-discriminant analysis (PLS-DA) score of biofluids of Jersey steers during moderate temperature period (MTP) and high temperature period (HTP) conditions. (A) PCA and (B) PLS-DA were performed for rumen, serum and urine samples from Jersey steers under MTP and HTP conditions. On the score plot, each point represents individual samples, with blue dot representing the MTP (rumen fluid and serum, $n = 8$; urine, $n = 4$), and red triangle representing the HTP ($n = 8$). In the PCA score plot, x-axis and y-axis represent the variance associated with PC 1 and 2, respectively. The PLS-DA score plot shaded ellipses represent the 95% confidence interval estimated from the score. The x-axis and y-axis represent the variance associated with component 1 and 2, respectively.

3.5. Analysis of DEGs in Jersey steers PBMCs under different THI

After transcript assembly, DEGs were analyzed based on read count values as a screening process. In addition, DEGs analysis was performed for comparatively studying DEGs within the immune cells and PBMCs, according to changes in Jersey steers under different THI condition. In this study, we determined that the genes with greater than twofold up- or down-regulation and $P < 0.05$, were significant DEGs. Through a quality check, we confirmed that a total of 24 DEGs were present in PBMC samples from Jersey steers. Hierarchical clustering analysis of the DEG list showed that the samples of Jersey steers were clustered according to environmental conditions (Fig. 6A). The clear segregation and clustering of the data indicated that distinct gene expression profiles existed, based on environmental changes. Volcano plots represented 12 upregulated and 12 downregulated genes (Fig. 6B). On collective assessment, only a small number of DEGs in PBMCs were identified in Jersey steers during MTP and HTP conditions.

3.6. Functional annotation of DEGs in PBMCs of Jersey steers during HTP

We further investigated the RNA-seq dataset using KEGG to identify the key immunological pathways of PBMCs of Jersey steers in response to HS. A total of 17 pathways were significantly different under MTP and HTP conditions ($P < 0.05$), and correction for multiple comparisons was performed by FDR (Fig. 7). We categorized five groups for 17 pathways, based on the following characteristics: 1) common cellular response: ErbB signaling pathway, Signaling pathways regulating pluripotency of stem cells, Phospholipase D signaling pathway, Calcium signaling pathway and Focal adhesion; 2) immune signaling pathway: MAPK signaling pathway, T cell receptor (TCR) signaling pathway, NF-κB signaling pathway and Cytokine–cytokine receptor interaction; 3) disease-related pathway: Pathways in cancer, Hepatitis B, Pancreatic cancer and Proteoglycans in cancer; 4) cellular metabolism: Purine metabolism and HIF-1 signaling pathway; and 5) others: AGE-RAGE signaling pathway in diabetic complications and Yersinia metabolism. The expression of several MAPK signaling pathway genes, including *FLNB* (−1.89), *TGFBR2* (−1.84), *CACNB3* (−1.69), and *PAK2* (−1.67) were downregulated by HS. Also, the cytokine–cytokine receptor interaction showed a downregulated pattern in HTP, such as expressions of *CXCL8* (−2.97), *IL12RB2* (−1.79), and *TGFBR2* (−1.84). In addition, NF-κB signaling pathway expression was also decreased in Jersey steer PBMCs during HTP, such as expressions of *CXCL8* (−2.97), *PLCG1* (−2.22), and *BIRC3* (−1.55). Furthermore, downregulation of genes associated with the HIF-1 signaling pathway resulted in reduced expression of functional molecular genes such as *PLCG1* (−2.22) and *STAT3* (−1.52). Interestingly, several pathways that were significantly different under HS shared common gene expression: *ERBB2*, *PLCG1*, and *CXCL8*. The integrated transcriptomic pathway represented the HIF-1, NF-κB, and phospholipase D signaling pathways, calcium signaling pathways, and cytokine–cytokine receptor interactions in Jersey steer PBMCs in

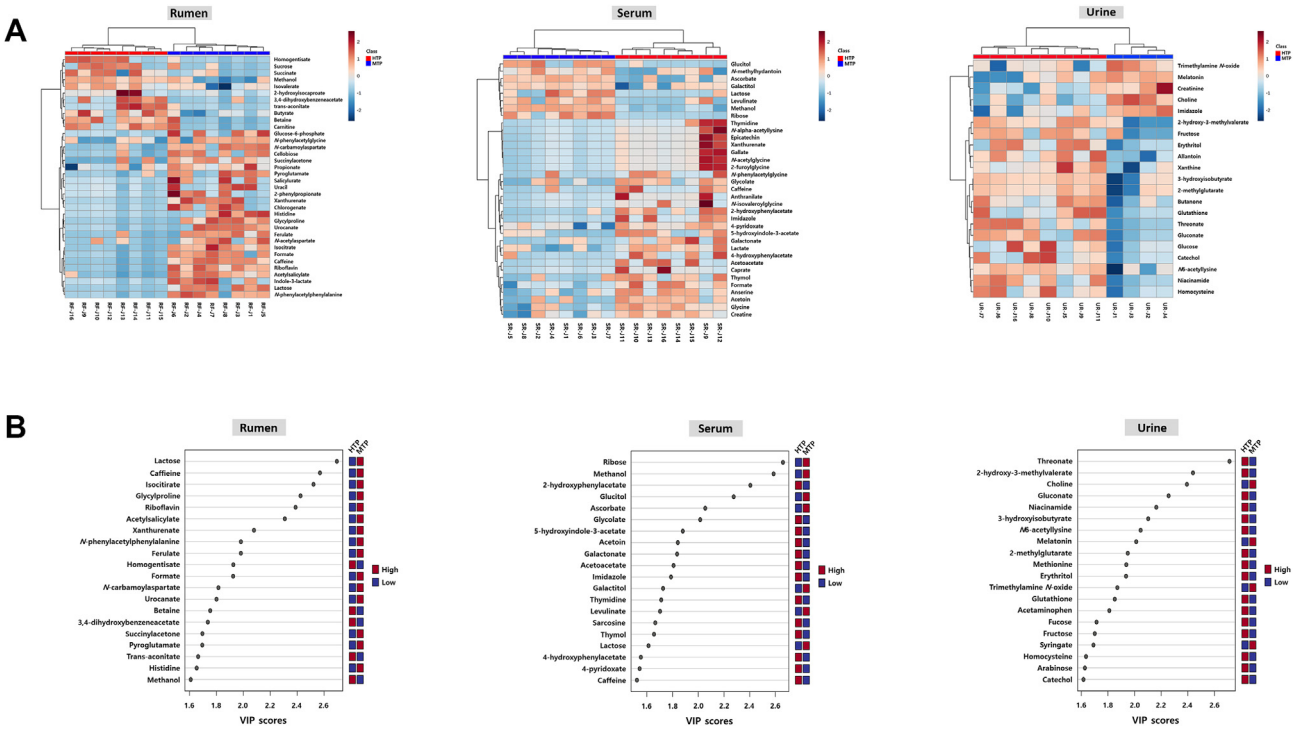

Fig. 3. Heatmap analysis and variable importance in projection (VIP) scores for differential metabolites in 3 biofluids of Jersey steers under moderate temperature period (MTP) and high temperature period (HTP) conditions were assessed. (A) Heatmap analysis of differential metabolites obtained by Student's *t*-test analysis ($P < 0.05$) of Jersey steers rumen fluid ($n = 8$/group), serum ($n = 8$/group) and urine (MTP, $n = 4$; HTP, $n = 8$). The red and blue colors in the plot describe high and low intensities, and the values ranges from −3 to +3. (B) VIP scores were assessed in rumen, serum and urine metabolite analysis. Top 20 metabolites among VIP score >1.5 were selected and ranked based on VIP scores. These represent up (Red) or down (Blue) regulation of metabolites in different environmental conditions. VIP scores were based on the partial least square-discriminant analysis model.

HTP (Fig. 8). Collectively, HS may affect the expression of specific genes that control the host cell signaling pathway by controlling the host's response to HS and the induced immune response.

4. Discussion

A combination of high temperature and humidity creates a hostile environment for cattle, as it may overwhelm the animals' capacity to dissipate heat and induce stress exceeding their physiological limits (Ronchi et al., 1997). Therefore, it is assumed that livestock placed under HS have a negative nutritional balance (NB), as determined by comparing the changes in their weight against that of animals in a thermoneutral zone (Yue et al., 2020). It is assumed that livestock placed under HS have a negative NB and undergo energy redistribution processes, such as raising blood insulin and protein catabolism in calves and heifers (Wang et al., 2020). Furthermore, heat-stressed cows fail to convert post-absorptive energetic metabolism, which means that HS can directly impact energetics (Wheelock et al., 2010). Beef cattle are considered less susceptible to HS than dairy cattle because of their higher THI threshold, caused by lower metabolic rate and body heat production (Nardone et al., 2010). However, the damage caused to beef cattle by HS due to global warming is increasingly becoming a serious cause for concern globally. The economic losses due to HS in the beef industry are tremendous, estimated at 20% of $1.69 to $2.36 billion (St-Pierre et al., 2003). Under these circumstances, a

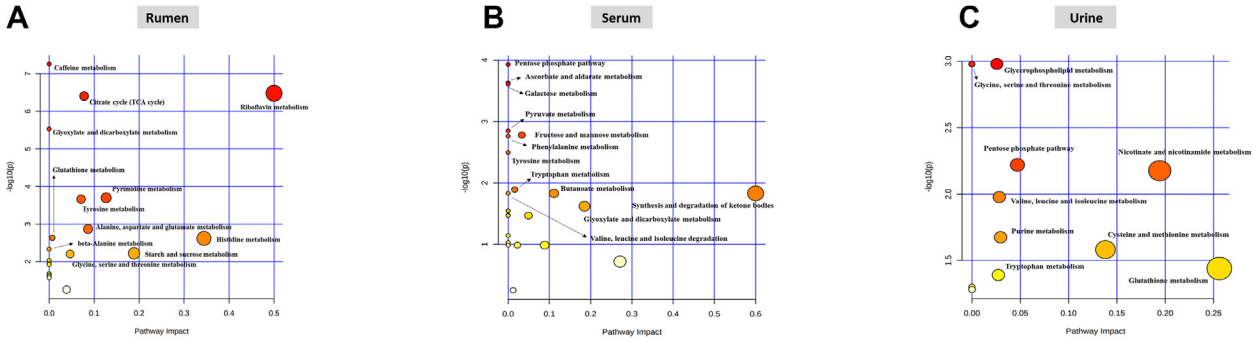

Fig. 4. The metabolome view map of the 3 biofluids. (A to C) Metabolic pathway mapping significantly differs in rumen fluid ($n = 8$/group), serum ($n = 8$/group) and urine (MTP, $n = 4$; HTP, $n = 8$) metabolites under moderate temperature period and high temperature period conditions. The pathway impact analysis was performed using Metaboanalyst 5.0 program. The x-axis and y-axis represent pathway impact value and −log 10 *P*-value, respectively. The results are presented graphically as a bubble plot. The darker color and larger size represent higher *P*-value from enrichment analysis and greater impact from pathway topology analysis, respectively.

Fig. 5. Integrated metabolomics pathway diagram of rumen fluid and serum from Jersey steers. The figure combines significantly different metabolic pathways of rumen fluid and serum under moderate temperature period (MTP) and high temperature period (HTP) conditions. The differentially expressed metabolites are illustrated by red or green boxes, which means up-regulated (red box) and down-regulated (green box) metabolite in HTP based on *P*-value ($n = 8$/group), respectively.

better understanding of the impact of HS on bovine physiology, metabolism and innovative strategies for countering the same are required to reduce economic loss and disease in beef cattle.

Several studies have reported that HS induces physiological parameters and alterations in the metabolic composition of biofluids. For example, HS noticeably changed the thermo-physiological characteristics of livestock, including rectal temperature, respiratory rate, and metabolic profile (Contreras-Jodar et al., 2019). Metabolomics has been used to study metabolites in biofluids to understand their physiological and biochemical status, with an increased interest in comprehensive and quantitative metabolic profiling (Psychogios et al., 2011). Under HS, Holstein cattle showed different metabolic patterns involved in proteolysis, gluconeogenesis, and fatty acid synthesis as compared to when placed under normal conditions (Yue et al., 2020). Volatile fatty acids (VFAs), such as acetate, propionate, and butyrate, are found in the rumen (MacLeod and Ørskov, 1984). Tajima et al. (2007) and Pragna et al. (2018) reported a decrease in rumen fluid total VFA, acetate, and acetate-to-propionate ratio and an increased butyrate concentration in Holstein heifers under HS. *Acetobacter* has the potential contribute to acetate by oxidizing sugars (Lyons et al., 2018). *Actinobacteria*, known as Gram-positive bacteria, can produce lactate and acetate as end-products using starch, polysaccharides and oligosaccharides (Ventura et al., 2007). Phylum to family Fibrobacteres group is associated with cellulolytic activity. Fibrobacteres are break-down plant-based cellulose in ruminants and contribute to acetate through fiber fermentation (Annison, 1970). Zhao et al. (2019) reported a decrease of *Acetobacter* population as well as acetate concentration in rumen fluid of lactating Holstein dairy cows under HS. Kim et al. (2020) reported that there were increased population of rumen fluid Fibrobacteres in Holstein dairy cows and decreased population of Actinobacteria in Jersey dairy cows under HS. In this study, acetate concentration was higher in the MTP, but not significantly different ($P > 0.05$). However, propionate and butyrate concentrations were altered by HTP. In addition, propionate concentration decreased, but butyrate

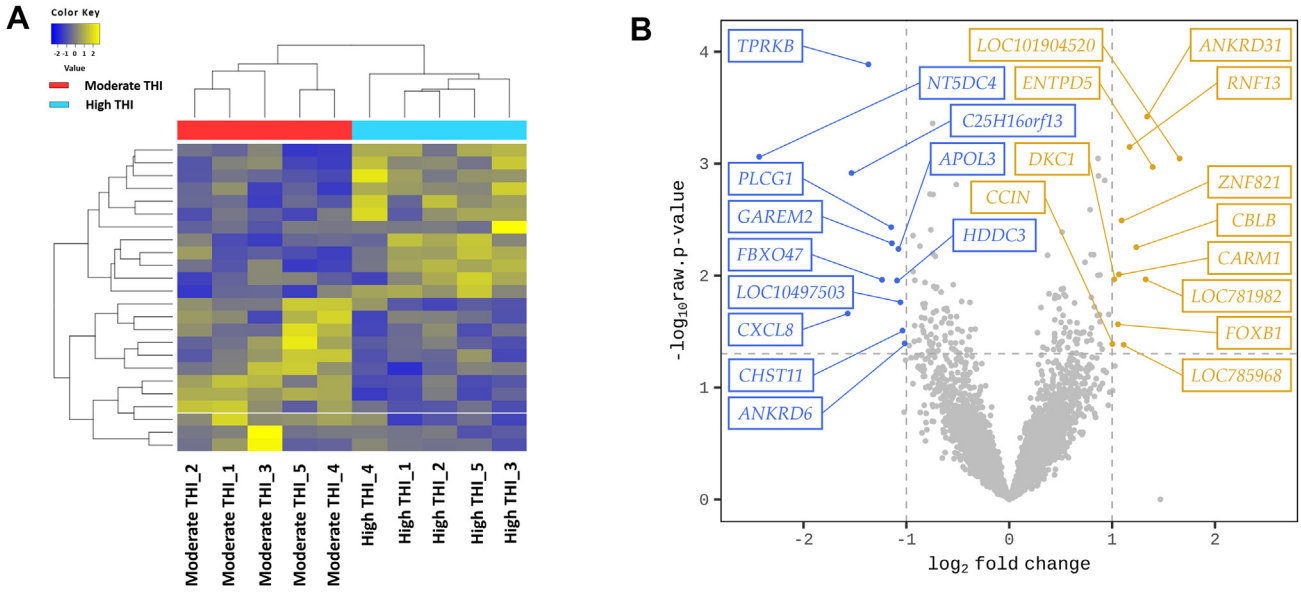

Fig. 6. The hierarchical clustering analysis and volcano plot for the significantly different in Jersey steer peripheral blood mononuclear cells (PBMCs) in different temperature humidity index (THI) conditions. (A) The heat map illustrates hierarchical clustering of differentially expressed genes (DEGs) in PBMCs of Jersey steers. The light blue boxes indicate an expression ratio less than mean, and the yellow boxes denote an expression ratio greater than the mean. Hierarchical clustering was performed using complete linkage method with Euclidean distances measures and those indicate higher similarities. (B) The DEGs in PBMCs of Jersey steers are presented in volcano plots ($P < 0.05$, fold-change < -2 or > 2). The top genes down-regulated (blue) or up-regulated (yellow) genes are highlighted ($n = 8$).

concentration significantly increased ($P < 0.05$ and $P < 0.01$, respectively), under the HTP. This result was similar to that reported by Bedford et al. (2020) and Islam et al. (2021), who studied effects of spring and summer periods, and pair-fed and HS groups in vivo, respectively. In summer, ruminants are more likely to experience ketosis due to increased maintenance requirements, thermoregulation and decreased feed intake (Gantner et al., 2016). Ketosis is classified into subclinical ketosis (SCK) (concentration of BHBA in blood between 1.2 and 1.4 mmol/L) and clinical ketosis (concentration of BHBA in blood between 2.6 and 3.0 mmol/L) (Duffield, 2000; Suthar et al., 2013). Doreau et al. (2001) reported that approximately 26% to 33% of butyrate absorbed by the rumen papillae was converted into BHBA in blood. *Butyrivibrio* in rumen fluid, are a butyrate-producer (Louis and Flint, 2017). In addition, Christensenellaceae which produce little amounts of butyrate as fermentation end products, probably play an important role in ketogenesis (Lima et al., 2015). Xiang et al. (2021) reported that *Butyrivibrio* and Christensenellaceae_R-7 abundance increases in the rumen fluid from SCK cows compared with healthy cows. In addition, Eom et al. (2021) reported higher concentrations of butyrate in the SCK group than in the healthy group. Therefore, butyrate is an energy source for ruminants. However, an excessively high concentration of butyrate in the rumen raises a concern for ketosis in ruminants. *Selenomonas ruminantium* can produce propionate through the decarboxylation process of succinate, or it can also produce propionate by fermenting carbohydrates or lactate (Van Soest, 2018). In this study, the succinate concentration in the HTP was significantly higher ($P < 0.05$) than that in the MTP, and propionate concentration in the MTP was significantly higher ($P < 0.05$) than that in the HTP. There is a possibility that HS may influence changes in *S. ruminantium* population in the rumen. In future studies, the rumen microbiome should be examined in order to understand ruminal physiology changes by HS together with metabolome changes. Formate is responsible for the toxicity observed in methanol poisoning (Barceloux et al., 2002). Formate toxicity includes optic nerve damage and metabolic diseases (Medinsky et al., 1997). In this study, the formate concentration in the HTP was significantly higher ($P < 0.01$) than that in the MTP. Hovda et al. (2005) reported that acidosis occurred only at higher concentrations of plasma formate. In addition, Yue et al. (2020) reported a higher concentration of formate in dairy cows under HS than in a thermos-neutral condition.

Serum and plasma lactate concentrations have been reported to be higher in animals under HS (Elsasser et al., 2009; Tian et al., 2015, 2016; Li et al., 2017). In this study, serum lactate concentration was significantly higher ($P < 0.01$) in the HTP than in the MTP. The source of this lactate is currently unknown, but it may have been derived from the gastrointestinal tract or skeletal muscles (Baumgard and Rhoads, 2013) where it would be the outcome of increased glycolysis to gain ATP in response to HS (Li et al., 2017). Li et al. (2016) reported that feeding cattle with high-concentrate diet resulted in higher metabolic activity, such as generation of various energy-generating molecules (e.g., glyoxylate and dicarboxylate metabolism) in the rumen. Doepel et al. (2002) reported that in lactating dairy cattle with a negative energy balance and higher glycine concentration in plasma, there was observed an association with the decomposition of muscle protein. Glycine is also involved in glyoxylate and dicarboxylate metabolism. Therefore, metabolites related to glyoxylate and dicarboxylate metabolism may have low concentrations in the rumen fluid during HTP and, conversely, high concentrations in serum. In this study, metabolite formate and isocitrate concentration in the HTP (associated with glyoxylate and dicarboxylate metabolism [$P < 0.0001$]) were significantly lower ($P < 0.05$) than those in the MTP in the rumen fluid. In addition, in respect of glyoxylate and dicarboxylate metabolism ($P < 0.05$), glycine concentrations in the HTP were significantly higher ($P < 0.01$) than those in the MTP in the serum. Succinate biosynthesis may be based on 3 pathways associated with carbon metabolism: the oxidative tricarboxylic acid (TCA) cycle, the reductive branch of the TCA cycle, and the glyoxylate pathway (Cheng et al., 2013). Succinate is the end product of fermentation when glycerol serves as a carbon source (Cheng et al., 2013). In addition, the biosynthesis of succinate as the main product from lactose is possible under anaerobic conditions. In this study, the lactose

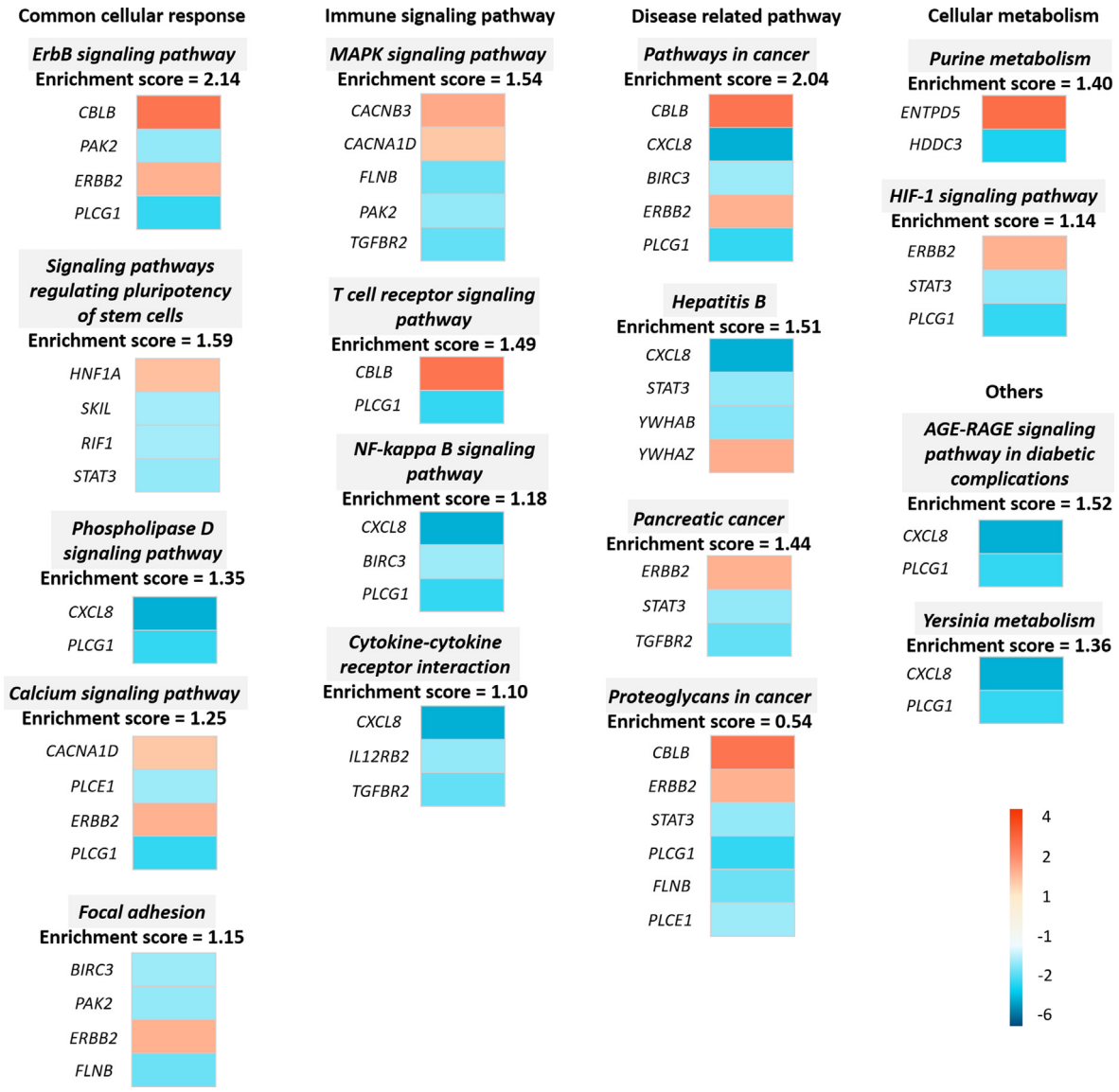

Fig. 7. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with differentially expressed genes of Jersey steer peripheral blood mononuclear cells (PBMCs) in different temperature humidity index conditions. KEGG pathways that discriminated under moderate temperature period and high temperature period conditions Jersey steers PBMCs and denote with each of enrichment scores below the terms. Blue color refers to significantly down regulated, while red color refers to significantly up regulation ($n = 8$).

concentration during the HTP was significantly lower ($P < 0.001$), and it showed the highest VIP score (2.69) during HTP than observed during the MTP. In contrast, succinate concentration in the HTP was significantly higher ($P < 0.05$) than that in the MTP. In addition, isocitrate (involved in the TCA cycle; the 3rd highest VIP score: 2.52) and formate (involved in glyoxylate and dicarboxylate metabolism; VIP score: 1.92) concentration in steers in the HTP were significantly lower ($P < 0.0001$ and $P < 0.001$, respectively) than that in the MTP. Therefore, it is possible that lactose, isocitrate, and formate may be used for succinate production as substrates in the rumen when under HS. These results suggest that lactose, isocitrate, and formate in rumen fluid may be considered as potential HS biomarkers.

In this study, amino acid metabolism was influenced by the HS environmental conditions. The capacity of amino acid catabolism is likely reinforced in the liver, and plasma amino acid concentrations are increased by nutrient deficiency (Stahel et al., 2014). The total amino acid concentration in the plasma is observed to have increased in lactating dairy cows under HS (Guo et al., 2018). Ai et al. (2015) and Guo et al. (2018) reported that the levels of amino acids (alanine, aspartate, glutamate, glycine, and valine) for gluconeogenesis were increased in heat-stressed cows. In this study, glycine and anserine concentrations were significantly increased ($P < 0.01$) during HTP. The amino acid derivative creatine is an important intermediate metabolite in energy reactions, and its phosphorylated form is an important metabolite that is a part of energy delivery system (Mercimek-Mahmutoglu et al., 2006). In the reverse reaction, a high-energy phosphate group, such as ATP, is transferred to creatine to form phosphocreatine and ADP (Wyss and Kaddurah-Daouk, 2000). In addition, arginine, glycine, and methionine are mainly synthesized in the liver. It is mainly used by tissues, absorbed into the blood, and excreted through urine (Poortmans et al., 2005). Additionally, creatine was identified as a potential biomarker for diagnosing HS in dairy cows, which may be attributed to the phosphocreatine in the muscle tissue that has been mobilized for energy supply (Tian et al., 2015). In this study,

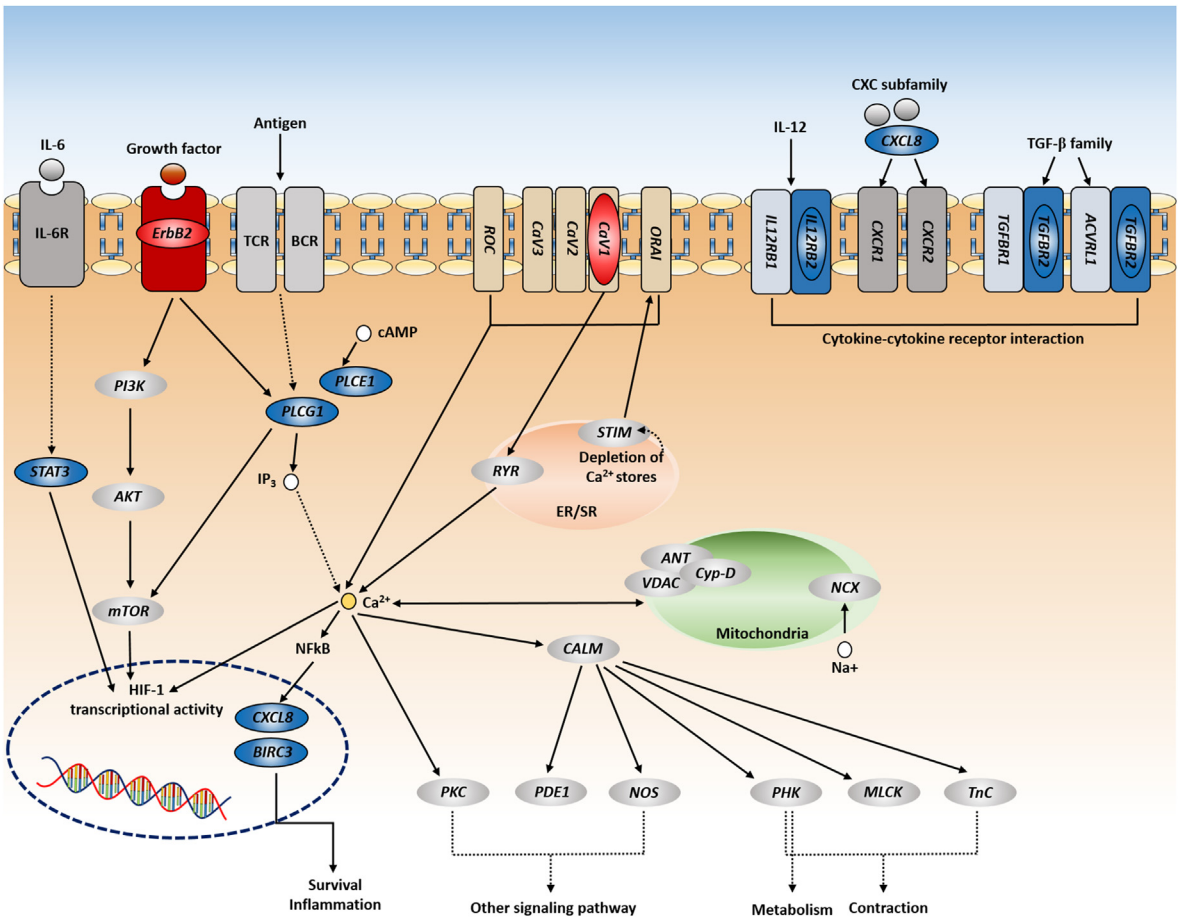

Fig. 8. Integrated transcriptomics pathway diagrams from Jersey steer peripheral blood mononuclear cells in different temperature humidity index conditions. The hypoxia-inducible factor-1 (HIF-1) signaling pathway, NF-κB signaling pathway, phospholipase D signaling pathway, calcium signaling pathway and cytokine–cytokine receptor interaction of Jersey steers are presented with identified pathway associated genes. Red color indicates an association with an increase in high temperature period (HTP). Blue color indicates an association with a decrease in HTP. Gray color or uncolored indicate no statistical significance ($n = 8$).

creatine concentration in HTP was significantly higher ($P < 0.01$) than that in the MTP.

Riboflavin functions in electron transfer reactions are related to energy, carbohydrate, lipid, and amino acid metabolism (Pinto and Zempleni, 2016). In particular, rumen microbial riboflavin synthesis is associated with dietary composition and increases with increasing levels of readily degradable carbohydrates and proteins (Beaudet et al., 2016; Castagnino et al., 2018). In this study, the riboflavin concentration in the MTP was significantly higher ($P < 0.0001$; VIP score: 2.39) than that in the HTP condition. In addition, riboflavin metabolism was significantly different ($P < 0.0001$; impact value: 0.5) observed in this study. Therefore, riboflavin in rumen fluid is considered a candidate HS biomarker.

Urine is also a useful source of biomarkers. In ruminants, urinary purine derivatives (PD) are by-products of nucleic acid digestion, and the majority of the nucleic acids that reach the intestines of a ruminant are derived from rumen microbes. There is a relationship between the quantity of purines and the microbial flow out of the rumen (Chen and Gomes, 1992; Fujihara and Shem, 2011). Chen et al. (1990) reported that increased excretion of PD in the urine indicates more microbial nucleic acids entering the small intestine. Urine PD consisted mainly of allantoin, uric acid, hypoxanthine, and xanthine. In this study, allantoin and xanthine (involved in purine metabolism) concentrations in the HTP were significantly higher ($P < 0.01$ and $P < 0.05$, respectively) than those observed in the MTP. Urine production in ruminants is measured to allow research on nitrogen balance and metabolites in the urine, such as creatinine (Whittet et al., 2019). Creatinine has been shown to be an effective marker for evaluating urine excretion in beef cattle (McDonald, 2003; Jardstedt et al., 2017). Muscle metabolism and creatinine excretion are directly associated with muscle mass and have a constant rate relative to the body weight (Brody, 1945; Gopinath and Kitts, 1984; Hayden et al., 1992). Gopinath and Kitts (1984) reported an increase in the urine creatinine excretion in growing beef cattle over time, indicating an increase in muscle mass with age. In this study, creatinine concentration in the MTP was significantly higher ($P < 0.05$) than that in the HTP. Mastitis causes significant economic damage to the dairy industry worldwide (Halasa et al., 2007; Nielsen and Emanuelson, 2013). Seasonal variation has also been reported in clinical mastitis in several studies, with a relatively higher incidence observed in the summer season (Green et al., 2006; Olde Riekerink et al., 2007). The excretion of glucose in the urine not only dissipates energy around the young calf, but it might also serve as an energy source for bacteria (such as *Streptococcus uberis*, a mastitis pathogen) in the urinary tract and cause urinary infection, bacteremia, and mastitis (Prajapati, 2019; Zwierzchowski et al., 2020). Therefore, glucose in urine is considered a candidate HS biomarker. In this study, the glucose concentration in the HTP was significantly higher ($P < 0.05$) than that in the MTP. As HS research using metabolites from urine is limited, these results will aid future studies on HS.

This study demonstrated the integrated metabolic pathways of rumen fluid and serum. First, in the rumen fluid, the detected metabolites, such as 3,4-dihydroxyphenylacetate, homogentisate, and succinate, showed an upregulated pattern during HTP. Interestingly, ruminal betaine, a metabolite of glycine, serine, and threonine metabolism, was upregulated in the HTP, and it was also connected to the upregulation of pyruvate metabolism in serum, specifically formate and lactate, in pyruvate metabolism. In addition, through aspartate and glutamate metabolism, downregulated isocitrate in the citrate cycle was associated with downregulated histidine, uracil, and riboflavin in β-alanine metabolism, pyrimidine metabolism, and riboflavin metabolism, respectively. Collectively, this suggests the possibility that upregulated or downregulated ruminal metabolites may contribute to the upregulation or downregulation, respectively, of other metabolic pathways in serum under HS environmental conditions.

RNA-seq is an innovative transcriptome analysis used to discover transcripts, analyze phenotypes, and map sequences onto the genome (Conesa et al., 2016). Transcriptome sequencing has considerable potential for identifying candidate genes associated with complex properties or diseases (Liu et al., 2020). HS-related changes in cattle have been documented to alter gene expression related to various metabolic activities involving lipid, carbohydrate, and amino acid metabolism, and it can be speculated that there are fundamental changes under HS environments that induce negative energy balance in cattle (Shahzad et al., 2015; Garner et al., 2017, 2020; Dado-Senn et al., 2018; Gao et al., 2019). In addition, relevant studies conducted on Holstein dairy cows have demonstrated an increase in immune activation and inflammation, suggesting a major role of TNF, IFN, S100A8, S100A9, and NF-κB (Gao et al., 2019). Other studies have reported that several genes in heat-stressed cattle are significantly enriched in immune effector processes, such as *OAS2*, *MX2*, *IFIT5* and *TGFB2* (Liu et al., 2020). In addition, Holstein bull calves showed hyperinsulinemia that was similar to an immune-stimulated status induced in response to HS through transcriptome analysis (Asea, 2008; Srikanth et al., 2017). By analyzing the PBMC transcriptome, it was revealed that Jersey dairy cows showed specific immunological changes under HS, such as within TNF and IL-17 signaling pathways (Kim et al., 2021).

To determine stress-related immunological changes, we used PBMCs, which are essential mediators in systemic immunity because HS-induced endocrinological changes would directly affect PBMCs in blood stream. For RNA seq. analysis, we obtained the PBMCs under different THI as reflecting the intensity of HS (normal vs. heat). The samples were collected in the middle of the day within MTP (THI = 64.92) and HTP (THI = 79.13). We assumed that samples were collected to assess the representative time slots as animals were sufficient exposed to different environmental condition and showed dramatic metabolic and physiological changes. However, the immunological analysis in serial time points in certain environmental conditions needs to be applied for comprehensive study in the future.

From the study, the significant KEGG pathways were identified to explore the essential immunological pathways of PBMCs under HS. Most of the genes in these pathways were downregulated under HS conditions. For example, the NF-κB signaling pathway, phospholipase D signaling pathways, and cytokine−cytokine receptor interaction were significantly altered in Jersey PBMCs by HS. NF-κB mediates critical changes in both innate and adaptive immune responses by regulating the early events in immune responses, including cytokines and other soluble factors that propagate and direct the initial step (Hayden et al., 2006). Interestingly, in a remarkable number of instances, HS has been implicated in the regulation of NF-κB signaling and altered immune homeostasis, making it more susceptible to pathogens. For example, constitutive NF-κB signaling plays a crucial role in the regulation of cell apoptosis by promoting the expression of related genes (Belardo et al., 2010; Liu et al., 2015; Tang et al., 2021). The phospholipase D signaling pathway regulates intracellular signaling and metabolic pathways, particularly in cells under stress conditions (Brown et al., 2017; Li et al., 2017). Phospholipase D has also been shown to play a positive role in TCR-mediated signaling and T cell activation (Reid et al., 1997; Cockcroft, 2001). Both phospholipase D and TCR signaling pathways have *PLCG1* as the common mediator and are key signaling molecules that are activated for TCR signaling transduction and TCR-mediated T cell selection and functions (Fu et al., 2012). The *PLCG1* gene was significantly downregulated during HTP, which is required for the regulation of intracellular signaling cascades. According to previous studies, *PLCG1* plays an important role in enhancing cell survival during the cellular response to HS (Bai et al., 2002; Kang et al., 2018). In the present study, the *CXCL8* gene expression was significantly decreased under HS conditions. It has been reported that *CXCL8* is primarily produced by neutrophils, macrophages, T lymphocytes, and endothelial cells and may control leukocyte trafficking during inflammation for homeostasis (Russo et al., 2014; Bie et al., 2019). Relevant studies conducted on the relationship between HS and immune responses have demonstrated that hypoxia induced by HS affects the expression of immune-associated genes, including *CXCL8* (Singh et al., 2008; Liu et al., 2015; Wang et al., 2021).

Recently, integrating study of transcriptome sequencing with metabolomics analysis has greatly increased the capacity of bioinformatics to explore significant and unveiled genes participating in the various pathways of specific biomarkers (Zhang et al., 2019). Crosstalk between metabolic and immunological pathways has become a key to understand and regulate the host defense (Halligan et al., 2016). As the interdependent relationship between metabolism and immunity has been gaining attention, it is necessary to investigate the bidirectional dialog to understand the host's physiological and immunological stance. In this study, we found a potential connection between the immune and metabolic pathways under HS conditions. For example, the NF-κB signaling pathway is known to regulate inflammation development and progression. According to the KEGG pathway analysis, the DEGs of Jersey steer PBMCs in the HTP showed downregulated expression in the NF-κB signaling pathway. Specifically, *CXCL8*, *BIRC3*, and *PLCG1* belong to the pathway that has been switched to decreased expression during HTP. Relevant studies have demonstrated that these downregulated genes are the main regulators of NF-κB signaling, as low gene expression is sufficient to modulate NF-κB signaling (Hoffmann et al., 2002; Asslaber et al., 2019). In addition, when analyzing the metabolites that were detected in the serum of Jersey steers, it was confirmed that the glycine from glyoxylate and dicarboxylate metabolism was upregulated under HTP. Interestingly, glycine is known to inhibit the activation of the NF-κB signaling pathway, which is in agreement with other studies showing that the regulation of specific genes and metabolites in charge of NF-κB activation finally results in weakening of the signaling pathway of the host under HS (Contreras-Nuñez et al., 2018; Arenas et al., 2020). In brief, as the key regulators for inhibition of HIF-1 and NF-κB signaling pathways were interrelated, it may suggest the possibility of an impact on not only the metabolic, but also on the immunological mechanism of the host.

5. Conclusions

In the present study, we observed differentially expressed metabolites and immune-related genes during MTP and HTP

conditions in order to understand the changes in metabolic and immunological responses in Jersey steers under HS. In this study, it was observed that several metabolite biomarkers could be used to diagnose HS in steers. Integrated analysis of the metabolomic pathway of rumen fluid and serum from Jersey steers revealed that ruminal metabolites significantly contribute metabolites in serum, confirming that these two biofluids are directly connected to modulate the host physiological and metabolic pathways. Although we could not reveal how each metabolite affects gene expression in immune cells, we believe that the altered metabolite profile in serum acts as one of the major regulatory factors for immune cell functions. Thus, the balance of metabolites and regulatory gene expression can lead to immune defense during HTP. This integrative analysis opens new avenues for research to understand the complex metabolic and immunological responses and their potential interactions in heat-stressed steers. Moreover, this analysis also helps to develop new diagnostic strategies to ameliorate the adverse effects of HS.

Author contributions

Jun Sik Eom: Conceptualization, Methodology, Software, Writing-Original Draft. **Da Som Park:** Conceptualization, Methodology, Writing-Original Draft, Visualization. **Sang Jin Lee:** Data curation, Software. **Bon-Hee Gu:** Supervision, Validation. **Shin Ja Lee:** Supervision, Software. **Sang-Suk Lee:** Methodology, Supervision. **Seon-Ho Kim:** Methodology, Investigation. **Byeong-Woo Kim:** Supervision. **Sung Sill Lee:** Validation, Resources, Project administration, Funding acquisition. **Myunghoo Kim:** Validation, Resources, Project administration, Funding acquisition.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.06.012.

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