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Running Title (within 10 words)	Metabolic and metataxonomic profile of stressed and recovered dairy cows
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Abstract

This study investigated the changes in rumen fermentation characteristics, blood parameters, and rumen microbial communities of Holstein dairy cows in the early lactation stage during heat stress conditions and subsequent recovery. This study aimed to fill the significant knowledge gaps regarding the recovery of dairy cattle from heat stress during the early stages of lactation. Metataxonomic analysis was used to identify potential biomarkers and metabolites associated with metabolic disease prediction. The temperature-humidity index was recorded on a dairy farm to define the heat stress and recovery periods. Using the Bray-Curtis dissimilarity index, principal coordinate analysis revealed that both the heat stress and recovery periods affected the overall composition of the rumen bacterial community. The first three principal coordinates explained 33.00%, 16.60%, and 11.60% of the total variation, indicating the significant ($p < 0.01$) influence of temperature changes on the dominance of rumen microbes and the rumen environment. However, alpha diversity measurements were unaffected in either period. Metataxonomic analysis (average relative abundance 2%) of cows in both periods revealed ten predominant genera: *Prevotella*, *Ruminococcus*, *Selenomonas*, *Gilliamella*, *Duncaniella*, *Succiniclasticum*, *Paraprevotella*, *Bacteriodes*, *Lentimicrobium*, and *Treponema*. During heat stress, significant alterations were observed in the levels of three organic acids, six fatty acids, and thirteen amino acids. Furthermore, heat stress caused a significant increase in blood serum HSP27 and HSP70 levels (both $p < 0.01$), whereas blood serum glucose ($p = 0.001$) and blood urea nitrogen ($p < 0.001$) decreased. Heat stress increased blood serum ketone concentrations, decreased cholesterol and blood urea nitrogen concentrations, and altered total protein, aspartate aminotransferase, and total bilirubin concentrations. The levels of blood serum minerals, such as calcium, phosphorus, and magnesium, as well as ruminal pH, ammonia-N, acetate, and butyrate, were not affected during either period. Heat stress influenced propionate ($p=0.006$) and total volatile fatty acids ($p=0.030$). Overall, heat stress during early lactation resulted in significant shifts within the rumen bacterial community structure, accompanied by corresponding changes in blood metabolite profiles.

Keywords (3 to 6): Early stage, Heat stress, Holstein dairy cow, Recovery period, Ruminal bacterial diversity

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Introduction

Global temperature amplitudes and incidences of extreme temperatures are expected to increase over the next century owing to anthropogenic climate change (1). Elevated temperatures can directly affect animal welfare by influencing physiological processes and disrupting symbiotic relationships between animals and other organisms, as indicated in previous studies (2,3). Among domestic animals, dairy cattle are particularly sensitive to heat stress, which presents a major challenge for the global dairy industry (4). The optimum thermoneutral zone for dairy cattle is 5 to 25 °C (5). When environmental temperatures exceed this range, cattle experience heat stress, triggering nonspecific immune responses as the body attempts to counteract the effects of stress (6). When the temperature-humidity index (THI) exceeds 72, these stress responses intensify as the body activates heat-coping mechanisms (7).

Heat stress in ruminants leads to a cascade of adverse effects, including reduced feed intake, poor gut motility, diminished rumination and ruminal contractions, inappetence, slow growth, increased disease risk, and even mortality (8–10). Furthermore, heat stress elicits significant physiological responses, notably altering numerous blood biochemical parameters, which further complicates the health management of dairy cattle (11–16). The transition period, defined as the phase encompassing three weeks before to three weeks after calving, is a critical time when dairy cows undergo substantial physiological and metabolic adjustments to meet the demands of late gestation, parturition, and the onset of lactation (17). During this period, high-producing dairy cows are particularly vulnerable to metabolic stress due to their elevated energy requirements, which predisposes them to periparturient diseases such as metritis, mastitis, laminitis, and ketosis (18). The additional burden of thermal stress during the transition period exacerbates these challenges, compromising animal welfare and increasing the risk of mortality, especially during summer (17,19,20).In the context of our study, the recovery period refers to the phase following the cessation of heat stress, during which dairy cattle begin to recuperate from the physiological strain induced by elevated temperatures. The recovery period is critical for understanding how cattle rebound from heat

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stress and how their physiological and metabolic parameters, including rumen function, blood composition, and overall health, return to baseline levels or stabilize (21–23). During this period, there is often a continued risk of subclinical or clinical ketosis, particularly if feed intake remains compromised, which can lead to conditions such as liver lipidosis (24). Additionally, oxidative stress and chronic hyperthermia during the transition period can result in reduced appetite and an increased risk of subclinical or acute ruminal acidosis (25). The rumen, home to a diverse and abundant microbial community, plays a crucial role in host metabolism and overall health. Heat stress has been shown to significantly alter the rumen microbial composition, although other factors such as nutritional management, grazing behavior, feeding strategies, environmental conditions, and host individuality also influence the assembly and function of this complex ecosystem (26–30). Heat stress typically results in a reduction in fiber-degrading bacteria such as *Ruminococcaceae* and *Fibrobacteres*, while promoting the growth of lactic acid-producing bacteria, including Lactobacillaceae and *Streptococcaceae* (31,32). This shift in microbial populations leads to decreased production of short-chain fatty acids (SCFAs), such as acetate, butyrate, and propionate, which are essential for the host's energy metabolism. Conversely, there is an increase in lactate concentrations, contributing to lower rumen pH and the potential development of subacute ruminal acidosis (SARA) (31,32).

Moreover, heat stress induces changes in metabolite profiles, with elevated levels of plasma non-esterified fatty acids (NEFAs) and blood urea nitrogen (BUN), reflecting a shift towards enhanced lipid metabolism and altered nitrogen utilization (33,34).The reduction in feed intake commonly observed during heat stress exacerbates these effects, further disrupting normal fermentation processes (17,35). Additionally, oxidative stress and inflammatory markers are elevated due to heat-induced damage to the rumen epithelium, compromising the ruminal barrier function and overall health (36).

Despite the extensive documentation of physiological and biochemical changes during heat stress and the transition period in dairy cattle, limited information is available regarding the recovery of dairy cattle from heat stress during early lactation, particularly in the transition from summer to autumn. Therefore, this study hypothesizes that during the recovery period following heat stress, Holstein dairy cows at early lactation will exhibit significant changes in rumen microbiota composition,

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blood biochemistry, and fermentation profiles, reflecting a process of physiological adaptation and stabilization.

Materials and Methods

All experimental procedures were conducted according to the Animal Experimental Guidelines of Sunchon National University Institutional Animal Care and Use Committee (SCNU-IACUC), South Korea. The SCNU-IACUC approved the experimental protocol (approval number: SCNU IACUC-2018-01).

Animals and Experimental Design

Twelve lactating Holstein dairy cows averaging 58 ± 23 days in milking (DIM), housed at the Woldeung dairy farm in Suncheon City, Jeollanam-do, South Korea, were used in the experiments. THI was monitored at the dairy farm during two periods: the heat stress period during the first week of August and the recovery period during the second week of October, during which collection and sampling were performed. The THI equation developed by the National Research Council (30) was used in this study, and the equation is as follows: THI = (1.8 x ambient temperature + 32) – ((0.55 – 0.0055 x relative humidity) x (1.8 x ambient temperature – 26)). The THI was 79.27 (average temperature of 27.68 ℃ and humidity of 80.54%) in the heat stress period and 61.58 (average temperature of 16.72 ℃ and humidity of 76.90%) in the recovery period. During the trial, dairy cows were fed twice daily at 06:00 and 17:00 h with a total mixed ration intended for early lactating cows (Table 1) and milked twice daily at 05:00 and 16:00 h using a milking machine.

Sample Collection

Before feeding, ruminal fluid samples were obtained via stomach intubation and stored in pre-chilled 50 mL conical tubes. Simultaneously, blood samples were drawn from the jugular vein using a 20 mL syringe and 19-gauge needle. Approximately 5 mL of blood was transferred into a vacutainer (Green Cross MS, Korea) containing K3-EDTA for the subsequent analysis of blood parameters. A blood specimen collection device (Becto Drive, Franklin Lakes, NJ, USA) and serum separator tubes (Belliver Industrial Estate, Belliver Way, Robrough, Plymouth, UK) were used to collect blood for complete blood count (CBC) and blood chemical composition analyses, respectively. All blood sample tubes were promptly placed in an

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insulated container with ice and transported to the laboratory for immediate processing.

16S rRNA Gene Sequencing and Analysis

DNA from rumen fluid was extracted by Macrogen (Macrogen Inc., Seoul, Korea) and sequenced, processed, and analyzed. Briefly, the V3–V4 regions of the 16S rRNA gene were amplified using PCR with primers that contained an ILMN pre-adapter + sequencing primer + specific locus primer: V3 (5′ - TCGTCGGCAGCGTC + AGATGTGTATAAGAGACAG + CCTACGGGNGGCWGCAG - 3′; forward) and V4 (5′ - GTCTCGTGGGCTCGGA + GATGTGTATAAGAGACAGG + ACTACHVGGGTATCTAATCC - 3′; reverse) according to the 16S Metagenomics Library Prep Guide (37) . The barcoded V3-V4 amplicons, present in equimolar amounts, were combined and subjected to paired-end sequencing using an Illumina MiSeq PE300 platform (Illumina Inc., San Diego, CA, USA). After sequencing, the raw data was categorized by sample using an index sequence, resulting in paired-end FASTQ files for each sample. The sequences were then demultiplexed, followed by the removal of barcodes and adaptor sequences utilizing the Cutadapt v3.2 software (38). Amplicon sequence variants (ASVs) were generated from the sequence reads following the established workflow of the Divisive Amplicon Denoising Algorithm 2 (DADA2) v1.18.0 (39). For paired-end reads, forward reads were truncated at 250 bp, and reverse reads at 200 bp, while sequences with an error threshold of ≥2 were excluded. The QIIME v1.9 program was employed to conduct a comparative analysis of the microbial community (40). Species-level annotations of the DNA sequences were performed using BLAST+ (v.2.9.0) against the NCBI 16S Microbial Reference Database(41). Raw data of taxonomic abundance (ASV) files were further processed by filtering the ruminal bacterial groups. The processed ASV files were then formatted as plain-text (.txt) tables with taxonomy labels before being uploaded to the web-based platform MicrobiomeAnalyst (42,43) (https://www.microbiomeanalyst.ca/ accessdate: 09/14/2024). A metadata file describing the group information (heat stress and recovery) was created in plain text format (.txt). The ASV table and metadata files were submitted to the MicrobiomeAnalyst web-based platform following the data analysis and visualization of Miguel et al (44) using the default setting. Data filtering was performed to remove low-quality features. A 10% prevalence threshold was applied to filter out low-count samples and the

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interquartile range was used to filter for low variance. The data were normalized, and total sum scaling was employed for data scaling. Alpha diversity parameters, including observed abundance-based coverage estimator (ACE), Chao1, Shannon, and Simpson indices, were calculated using the t-test or analysis of variance (ANOVA). Beta diversity profiling was conducted using principal coordinate analysis (PCoA) with the Bray-Curtis index used for assessment.

Metabolite Analysis

Gas chromatography-tandem mass spectrometry

Serum metabolomic profiling was carried out to assess the presence of organic acids (OAs) and fatty acids (FAs) in multiple reaction monitoring mode using gas chromatography-tandem mass spectrometry (GC-MS/MS) in multiple reaction monitoring (MRM). This method was performed as previously described (45).

Sample preparation for simultaneous profiling analysis of OAs, AAs, and FAs in serum

A previously described protocol enabled the simultaneous profiling analysis of oxysterols (OAs), bile acids (AAs), fatty acids (FAs), and methoxime/tert-butyldimethylsilyl (MO/TBDMS) derivatives (46,47).

To summarize the sample preparation procedure, proteins were removed from plasma samples. Acetonitrile (150 μL) was added to 50 μL of plasma, which contained 0.1 μg of 3,4-dimethoxybenzoic acid and lauric-d2-acid as internal standards (ISs). The resulting supernatant, subsequent to centrifugation, was mixed with 800 μL of distilled water. Each aliquot solution was then adjusted to a pH of ≥ 12 using 5.0 M sodium hydroxide. Subsequently, the solution was converted into the MO derivative by reacting with methoxyamine hydrochloride at 60 °C for 60 min.

The resulting aqueous phase, now in sequential MO derivative form, was acidified (pH ≤ 2.0) using 10% sulfuric acid, saturated with sodium chloride, and extracted using ethyl acetate (2 mL), a mixture of ethyl acetate (2 mL) and diethyl ether (3 mL), and diethyl ether (3 mL), respectively. The extracts were evaporated to dryness under a gentle nitrogen stream. The dry residues, containing OAs and FAs, were further subjected to reaction at 60 °C for 60 minutes with triethylamine (5 μL), toluene (10 μL), and N-Methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (20 μL) to form the TBDMS derivative.

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To establish calibration samples for quantification analysis, samples containing ISs, OAs, and FAs at various concentrations (ranging from 0.01 to 5.0 μg/mL) using the sequential MO/TBDMS derivatives were prepared, following the described procedure. All samples were individually prepared in triplicate and subsequently analyzed using gas GC-MS/MS in MRM modes.

Liquid chromatography-tandem mass spectrometry

In preparation of the serum samples, a combination of 20 μL of serum with 13C1 phenylalanine as the internal standard (IS) at a concentration of 50 ng and 60 μL of acetonitrile (ACN) (60 μL) is required. Following centrifugation at 12,300 × g for 3 minutes, supernatants were clarified through 0.22 μm Spin-X centrifuge filters (Costar) for subsequent LC-MS/MS analysis. Aliquots (1 μL) of the clarified supernatants were injected into an LCMS-8050 system (Shimadzu) using an autosampler.The method employed in this study was aimed at profiling 49 AAs. Calibration samples containing the IS and AAs at various concentrations (0.005 to 2.0 μg/mL) were prepared and subjected to quantification analysis following the described procedure.

Chromatographic separations were performed using an Intrada AA column (50 mm x 3.0 mm, 3 μm) at a flow rate of 0.6 mL/min. The mobile phase comprised solvent A (ACN/THF/25 mM ammonium formate/acetic acid = 9/75/16/0.3, v/v/v/v) and solvent B (ACN/100 mM ammonium formate = 20/80, v/v). The gradient elution started at 0% B for 2.5 minutes, increased to 17% B over 6.5 minutes, reached 100% B at 10 min, returned to 0% B at 12 min, and concluded with a 3-minute re-equilibration period.

For the MS/MS analysis, electrospray ionization mode was employed. The column oven, autosampler, interface, desolvation line, and heat block were set at temperatures of 40, 4, 200, 200, and 300 °C, respectively.. The nebulizing, drying, and heating gases operated at flow rates of 3.0, 10.0, and 10.0 L/min, respectively. The collision-inducing dissociation gas pressure was maintained at 270 kPa.

Star pattern recognition analysis and multivariate statistical analysis

The concentrations of OAs, AAs, and FAs in plasma samples from both cohorts were quantified using calibration curves and expressed as percentages (%). To compare the metabolite levels between the groups, the values were standardized against the mean values of the corresponding recovery group. Each value is graphically represented as a spoke emanating from a a common central point. Linking the outer

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ends of these spokes (18 for OAs, 23 for AAs, and 22 for FAs) resulted in the formation of star-shaped patterns using Microsoft Excel.

For the multivariate analysis, the data were log10-transformed and Pareto-scaled. Principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and heat maps were generated using MetaboAnalyst. The soundness of the PLS-DA model was ascertained based on statistical parameters such as the correlation coefficient (R^2) and cross-validation correlation coefficient (Q^2), following the approach described in previous studies (46–49).

Heat Shock Protein (HSP) Analysis

Serum concentrations of HSP27, HSP70, and HSP90 were determined using commercially available, bovine-validated ELISA kits from MyBiosource (San Diego, CA, USA) following the manufacturer's protocol.***Blood Biochemistry Analysis***

Serum isolation was achieved via centrifugation of collected whole blood at 4000 rpm for 10 minutes at 4 °C. The resultant supernatant, designated serum, was carefully transferred and stored at -20 °C for subsequent analyses.Serum samples were analyzed using the Catalyst One Chemistry Analyzer (IDEXX Laboratories, Inc., USA) to quantify a panel of biochemical markers, including aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium, cholesterol, magnesium, inorganic phosphate, total bilirubin, and total protein.Glucose and ß-ketone test strips were used to determine blood glucose and ketone levels.

Furthermore, a subset of blood serum samples was forwarded to the Department of Pharmacy, Sunchon National University, South Korea, for additional metabolomic analysis.

CBC analysis

Freshly collected blood samples were expeditiously conveyed to the laboratory for comprehensive CBC analysis. Utilizing a hematology analyzer from INDEXX Laboratory, Inc. (USA), the CBC parameters examined encompassed the total erythrocyte count (RBC), hematocrit value (HCT), hemoglobin concentration (HGB), mean erythrocyte volume (MCV), mean hemoglobin content per red blood cell (MCH), mean hemoglobin concentration of erythrocytes (MCHC), red blood cell distribution width (RDW), reticulocyte count (RETIC), total leukocyte count (WBC), percentage of neutrophils (%NEU), percentage of lymphocytes (%LYM), percentage of monocytes (%MONO), percentage of eosinophils (%EOS),

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225 percentage of basophils (%BASO), absolute neutrophil count (NEU), absolute lymphocyte count (LYM),
226 absolute monocyte count (MONO), absolute eosinophil count (EOS), absolute basophil count (BASO),
227 total platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), and platelet crit
228 value (PCT).

229 ***Rumen Fermentation Parameters Analysis***

230 Upon the collection of rumen fluid samples, various parameters such as pH, volatile fatty acids
231 (VFA), and ammonia nitrogen (NH_3-N) were analyzed. The pH was prompty measured using a pH and
232 multiparameter instrument from Mettler-Toledo GmbH (Analytical Im Langacher 448606, Greifensee,
233 Switzerland).

234 The treatment of the rumen fluid samples involved storing them at -80 °C in 1.5 mL cryotubes,
235 followed by thawing at room temperature and centrifugation at 13,000 rpm for 15 minutes at 4 °C using a
236 Micro 17TR centrifuge from Hanil Science Industrial, Incheon, Korea.

237 Subsequently additional rumen fluid samples were also collected, stored under similar conditions,
238 and then subjected to the same thawing and centrifugation process. The resulting supernatant was utilized
239 for NH_3-N and VFA analyses. NH_3-N concentration measurement employed the methods of Chaney and
240 Marbach (50) and a Libra S22 spectrophotometer from Biochrom Ltd., Cambridge CB4 0FJ, England. VFA
241 analysis was performed using high-performance liquid chromatography (Agilent Technologies 1200 series,
242 Tokyo, Japan) with a UV detector set at 210 and 220 nm, and a Metacarb87H column from Agilent
243 Technologies, Minnetonka, MN, USA. A 0.0085 N H2SO4 buffer solution was used at a flow rate of 0.6
244 mL/min, and the column temperature was maintained at 35 °C (51).

245 ***Statistical Analysis***

246 Statistical analysis was performed using SAS version 9.1 statistical package (SAS Institute, Cary,
247 NC, USA) to assess the differences in rumen fermentation parameters, blood parameters, and relative
248 abundance between the two periods. A general linear model and ANOVA were employed for data analysis.
249 Duncan's multiple range test was utilized to identify significant differences between the two periods.
250 Statistical significance was set at $p < 0.05$ means. Permutational multivariate analysis of variance
251 (PERMANOVA) was used to assess the statistical differences between the clusters of the two groups. At

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the species level, linear discriminant analysis (LDA) effect size (LEfSe) was performed, employing a false discovery rate (FDR)-adjusted p-value cutoff significance of 0.05 and a log LDA score of 2.0. To determine significant differences between the normalized metabolites of the two cohorts, the Mann-Whitney U test, a nonparametric statistical method, was employed

Results

Ruminal Bacterial Diversity

The sequencing of 16S rRNA genes from the rumen fluid yielded a total of 756,554 reads, which were subsequently rarefied across all samples to match the depth of the sample with the lowest read count. The PCoA plot showed distinct cluster samples from the heat stress and recovery periods, indicating significant differences in bacterial community composition between these two periods (Figure 1). Principal coordinates 1, 2, and 3 accounted for 33.10%, 19.10%, and 12.30% of the total variation, respectively. Alpha diversity metrics are presented in Figure 2. There were no significant differences between the heat stress and recovery groups in ACE, observed species, and Chao1, Shannon, and Simpson indices using a t-test ANOVA. There were 366 shared species between the heat stress and recovery groups (Figure 3), and 172 and 118 unique species for the heat stress and recovery groups, respectively.

Ruminal Bacterial Composition

In this study investigating the influence of early lactation heat stress on the ruminal microbial community, a taxonomic analysis of rumen bacterial populations was performed.. Figure 4 presents the relative abundance comparisons of ruminal microbiota composition at the phylum and genus levels. The analysis revealed the presence of three predominant phyla (with an average relative abundance ≥ 2%) in the rumen during both the heat stress and recovery periods: *Bacteroidetes* (57.08% during heat stress and 55.71% during the recovery period), *Firmicutes* (31.97% and 27.41% during heat stress and recovery period, respectively), and *Proteobacteria* (5.87% and 10.45% during heat stress and recovery period, respectively, $p < 0.05$). These findings provide insights into the potential impact of heat stress on the composition of ruminal microbiota at different taxonomic levels. Taxonomic analysis at the genus level identified ten predominant genera (with an average relative abundance ≥ 2%) during both the heat stress and recovery

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periods. These genera play a significant role in the ruminal microbiota composition and include *Prevotella* (42.24% and 39.80% in heat stress and recovery period, respectively), *Ruminococcus* (4.75% and 5.12% in heat stress and recovery period, respectively), *Selenomonas* (4.34% and 4.98% in heat stress and recovery period, respectively), *Gilliamella* (1.89% and 7.30% in heat stress and recovery period, respectively, $p < 0.01$), *Duncaniella* (3.66% and 2.93% in heat stress and recovery period, respectively), *Succiniclasticum* (4.06% and 1.85% in heat stress and recovery period, respectively), *Paraprevotella* (2.65% and 2.57% in heat stress and recovery period, respectively), *Bacteriodes* (2.38% and 2.42% in heat stress and recovery period, respectively), *Lentimicrobium* (1.57% and 2.58% in heat stress and recovery period, respectively, $p < 0.05$), and *Treponema* (1.09% and 2.14% in heat stress and recovery period, respectively, $p < 0.01$).

LEfSe was done to determine the singular effect of heat stress and the recovery period on the rumen microbiota (Figure 5). During heat stress, *Blautia luti*, *Schwartzia succinivorans*, *Ethanoligenens harbinense*, *Faecalicatena orotica*, *Breznakibacter xylanolyticus*, *Aestuariispira insulae*, *Butyrivibrio proteoclasticus*, *Hallella seregens*, *Olsenella profusa*, and *Prevotella buccalis* were the top ten taxa detected at species level. During the recovery period, *Gilliamella bombicola*, *Gilliamella bombi*, *Lentimicrobium saccharophilum*, *Paludibacter propionicigenes*, *Treponema bryantii*, *Prevotella scopos*, *Anaerocolumna cellulosilytica*, *Treponema saccharophilum*, *Vallitalea pronyensis*, and *Prevotella marshii* were the top ten taxa identified to be enriched at species level.

Metabolic Profiling Analysis of 57 Metabolites in Blood Serum

The metabolic profiling of blood serum using GC-MS/MS MRM mode revealed significant differences in 22 metabolites between the heat stress and recovery periods. Among the organic acids (OAs), succinic acid ($p < 0.01$), malic acid ($p = 0.021$), and hippuric acid ($p = 0.014$) exhibited significantly higher levels during heat stress. In the fatty acids (FAs) category, myristic acid ($p < 0.01$), palmitoleic acid ($p = 0.021$), palmitic acid ($p < 0.01$), and oleic acid ($p < 0.01$) were found to have elevated levels during heat stress. In contrast, docosatetraenoic acid ($p = 0.030$) and 14-methylpentadecanoic acid ($p < 0.01$) showed significantly reduced levels during this period.

Among the amino acids (AAs), glutamic acid ($p < 0.01$), proline ($p < 0.01$), hydroxyproline ($p < 0.01$), creatinine ($p < 0.01$), 1-methylhistidine ($p < 0.01$), and pyroglutamic acid ($p < 0.01$) were

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significantly elevated during heat stress. Conversely, tryptophan ($p < 0.01$), tyrosine ($p = 0.014$), leucine ($p < 0.01$), isoleucine ($p < 0.01$), valine ($p < 0.01$), threonine ($p = 0.017$), and arginine ($p < 0.01$) were significantly lower in heat stress compared to the recovery period.

Star Pattern Recognition Analysis

The relative compositions of the 57 metabolites in blood serum were normalized by comparison with the mean values of the recovery group. Among the metabolites analyzed, several showed significant differences during the heat stress period compared to the recovery group. For organic acids (OAs), significant increases were observed in succinic acid (No. 7), malic acid (No. 9), and hippuric acid (No. 12) during heat stress. In the fatty acids (FAs) category, elevated levels were noted in myristic acid (No. 13), palmitoleic acid (No. 14), palmitic acid (No. 15), oleic acid (No. 18), and 14-methylpentadecanoic acid (No. 29). However, docosatetraenoic acid (No. 24) showed a lower level during heat stress. Among the amino acids (AAs), significant decreases were observed in tryptophan (No. 31), tyrosine (No. 33), leucine (No. 34), isoleucine (No. 36), valine (No. 37), and arginine (No. 56) during the heat stress period. Conversely, the levels of glutamic acid (No. 39), proline (No. 41), hydroxyproline (No. 42), threonine (No. 43), creatinine (No. 48), 1-methylhistidine (No. 51), and pyroglutamic acid (No. 57) were significantly elevated during heat stress.

PCA and PLS-DA in Differentiating Heat Stress and Recovery Phases

The PCA score plot exhibited a cumulative variance of 45.2%, with the first principal component explaining 26.1% and the second explaining 19.1% of the total variation. However, no clear separation was observed between the heat stress and recovery periods (Figure 7). In contrast, the PLS-DA of the 57 metabolites demonstrated a complete separation between the heat stress and recovery periods (Figure 7). The first and second components of PLS-DA explained 24% and 15.8% of the total variation, respectively. PLS-DA highlighted several metabolites, including tryptophan, creatinine, hydroxyproline, leucine, glutamic acid, arginine, isoleucine, valine, pyroglutamic acid, oleic acid, 14-methylpentadecanoic acid, 1-methylhistidine, hippuric acid, proline, and palmitic acid, which ranked among the top 15 in terms of variable importance in the projection (VIP) scores used to distinguish between heat stress and recovery periods (Figure 8). Furthermore, heatmap analysis was conducted using the top 25 metabolites from the

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PLS-DA analysis, revealing a distinct separation between the heat stress and recovery periods (Figure 9), providing further evidence of their differentiation.

HSP

Blood serum concentrations of HSP27, HSP70, and HSP90 during the heat stress and recovery periods are shown in Figure 10. Blood serum HSP27 and HSP70 levels increased significantly ($p < 0.01$) by 2,686.00 and 2.25 ng/mL, respectively, during heat stress. However, HSP90 levels were not significantly different between the heat stress (14.02 pg/mL) and recovery (12.91 pg/mL) periods.

Effect of Heat Stress on Blood Biochemical Parameters

The biochemical parameters of the blood serum during the heat stress and recovery periods are presented in Table 3. The blood serum glucose was significantly lower ($p = 0.001$) in the heat stress period than during the recovery period, at 58.33 and 69.30 mg/dL, respectively. The ketone body concentration denotes the quantity of ketone bodies in the bloodstream, which was assessed in this study. The ketone body concentration in blood serum was significantly higher ($p = 0.005$) during HSheat stress (0.79 mmol/L) than during recovery (0.55 mmol/L). The BUN levels were significantly lower ($p < 0.001$) during the heat stress period (8.25 mg/dL) than during the recovery period (12.90 mg/dL). For calcium and phosphorus levels in the blood, the heat stress period had lower concentrations for both minerals (8.53 and 6.41 mg/dL, respectively) than during the recovery period (8.86 and 6.51 mg/dL, respectively) but not significantly different. In contrast, blood magnesium levels increased during the heat stress period; however, the difference was not statistically significant. The total protein concentration in the blood serum was significantly higher ($p = 0.002$) in heat stress cows (10.21 g/dL) than in recovery cows (8.02 g/dL). AST levels were significantly higher during the heat stress period (107.08 U/L) than during the recovery period (86.60 U/L). In addition, total bilirubin levels were significantly higher ($p = 0.010$) during the heat stress period than during the recovery period. Serum cholesterol levels were lower during the heat stress period (162.833 mg/dL) than during the recovery (199.900 mg/dL).

Effect of Heat Stress on CBC

The CBC results for animals under heat stress and recovery conditions are shown in Table 4. The RBC and the ratio of HCT to erythrocyte count, representing the total blood volume, HGB, MCH, and mean

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MCHC, were higher during the heat stress period than during the recovery period, although the differences were not statistically significant. Specifically, the heat stress period exhibited values of 6.06 M/μL for erythrocyte count, 0.29% for HCT (erythrocyte ratio), 9.75 g/dL for HGB, 16.13 pg for MCH, and 34.26 g/dL for MCHC. In contrast, the recovery period had values of 5.93 M/μL for erythrocyte count, 0.28% for HCT to erythrocyte ratio, 9.42 g/dL for HGB, 15.83 pg for MCH, and 33.57 g/dL for MCHC. However, the MCV in the total sample, the degree of variation in size of the erythrocyte population (%), and RETIC were higher during recovery 47.17 fL, 0.252%, and 1.17 K/ μL, respectively) than during heat stress (47.15 L, 0.24%, and 1.03 L/ μL) but the differences were not significant. The WBC, %MONO, NEU, LYM, and MONO were higher during heat stress (12.76 K/ μL, 0.14%, 6.22 K/ μL, 4.22 K/ μL, and 1.70 K/ μL, respectively) than during recovery (11.66 K/ μL, 0.14%, 5.63 K/ μL, 3.96 K/ μL, and 1.70 K/ μL, respectively), but not significantly different. In addition, the %EOS and EOS were significantly higher during heat stress (0.05% and 0.58 K/ μL, respectively) than during recovery (0.03% and 0.34 K/ μL, respectively). However, the %NEU, %LYM, %BASO, and BASO count were higher during recovery (0.48%, 0.35%, 0.003%, and 0.03 K/ μL, respectively) than during heat stress (0.46%, 0.35%, 0.002%, and 0.03 K/ μL) but the differences were not significant ($p=0.756$, $p=0.928$, $p=0.432$, $p=0.735$, respectively).

During heat stress, the PLT and %PCT value were higher (428.00 K/ μL and 0.004%, respectively) than during recovery (293.50 K/ μL and 0.003%), but not significantly different. In contrast, MPV and PDW; the degree of variation in size of the platelet population, was significantly higher during the recovery period, at 10.58 fL and 7.80 fL, than during the heat stress period, at 9.63 fL and 7.08 fL, respectively.

Rumen Fermentation Parameters

A comparison of rumen fermentation parameters between the heat stress and recovery periods in Holstein cows during early lactation is shown in Table 5. The pH values at the two -time points were not significantly different (heat stress= 6.33, recovery= 6.39) but were lower during the heat stress period. NH_3-N during the heat stress period (3.64 mg/dL) was higher than during the recovery period (2.84 mg/dL); however, the difference was not significant. The concentration of acetate and butyrate during heat stress (57.71 and 18.94 mmol/L, respectively) and recovery (53.22 and 16.99 mmol/L, respectively) did not differ from each other but were higher during heat stress. However, propionate and total VFA were significantly

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higher in heat stress (27.76 and 104.50 mmol/L, respectively) than in recovery (20.32 and 90.52 mmol/L). A significant increase in the propionate levels resulted in a reduction in the A/P ratio during heat stress. Meanwhile, this proportion increased ($p = 0.053$) in the A/P ratio during recovery.

Milk Yield and Composition

Table 6 presents a comparative analysis of milk yield and composition in Holstein cows under heat stress conditions and subsequent recovery.Milk yield (L/d), milk fat (%), and milk protein (%) at the two time points were not significantly different. The solid non-fat (SNF) during the heat stress (8.72%) was significantly lower than that during the recovery (9.39%). Furthermore, milk urea nitrogen was significantly lower during the heat stress (6.30%) than during the recovery (10.14%).

Discussion

The composition and diversity of ruminal microbiota are crucial factors that can significantly influence rumen function (52–54). PCoA analysis revealed distinct clustering of bacterial communities during heat stress compared to the recovery period, indicating a significant shift in bacterial composition.This suggests that heat stress affects the composition of the bacterial community and that there is a shift in the community structure during recovery. Previous studies highlighted the importance of microbial diversity and richness in this context. In our study, we assessed alpha diversity indices, including observed species, Chao1, Shannon, and Simpson indices, and found no significant differences between the heat stress and recovery groups. This stability in diversity indicates that the rumen microbial ecosystem retains its core functional capabilities despite environmental challenges. Functional redundancy in microbial ecosystems allows for the preservation of essential metabolic activities even when microbial diversity does not change significantly (55). Previous studies also suggest that microbial stability may play a beneficial role in maintaining rumen functionality during short-term heat stress (53). Consistent with previous research, we observed that the three dominant phyla in the rumen were Proteobacteria, Firmicutes, and Bacteroidetes (52). These phyla were also predominant in the rumen of dairy cattle during both heat stress and recovery periods. At genus level, during heat stress, we observed significant changes in the relative abundances of rumen genera, including *Gilliamella, Lentimicrobium,* and *Treponema*, which may

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play crucial roles in ruminal digestion, metabolic processes, and immune responses. *Gilliamella*, though primarily associated with insect guts, has also been identified in the rumen, with species like *Gilliamella bombicola* contributing to the fermentation of complex carbohydrates into volatile fatty acids (VFAs), particularly acetate and propionate, which serve as essential energy sources for ruminants and support glucose synthesis and energy metabolism (56).While the immune interactions of *Gilliamella* in the rumen remain understudied, microbes involved in VFA production indirectly support immune function by promoting gut health and providing metabolic substrates, such as butyrate, which help maintain gut barrier integrity (34). *Lentimicrobium*, a genus within the phylum Bacteroidetes, plays also a significant role in the anaerobic fermentation processes in the rumen by contributing to the breakdown of polysaccharides and complex carbohydrates in plant materials (56). *Lentimicrobium* may support immune functions by producing metabolic byproducts like acetate and butyrate, which help maintain ruminal and intestinal lining integrity (57). Disruptions in its abundance may impair microbial balance, leading to inflammation and immune dysfunction (58). *Treponema* plays a key role in producing short-chain fatty acids (SCFAs) like butyrate, which are important not only as an energy source but also for maintaining gut integrity and overall health (59). Several *Treponema* species are known to have immune-modulating effects due to their role in producing butyrate, which has anti-inflammatory properties(60). Butyrate helps maintain the integrity of the gut epithelial barrier, preventing pathogen translocation and modulating the immune response (59). A decrease in Treponema abundance may lead to reduced butyrate levels, weakening gut barrier functions and increasing inflammation risks (60).

In our study, LEfSe analysis identified ten species that exhibited reduced abundance during the heat stress period. Notably, *Paludibacter propionicigenes* and *Treponema saccharophilum* decreased during heat stress, indicating their potential roles in rumen fermentation. *Paludibacter propionicigenes* is known for saccharolytic fermentation, producing propionate and acetate as major end-products.The altered proportion of this bacterium during heat stress suggests that its reduced abundance may impact the fermentation of diverse sugars, ultimately affecting propionate production in the rumen. Given that propionate (mmol) was significantly higher during the heat stress period, the altered abundance of *Paludibacter propionicigenes* might reflect adaptive changes in the microbial population that support an increased production of

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propionate.

Metabolomic analysis was conducted on the blood serum of Holstein dairy cows during the heat stress and recovery periods using GC-MS/MS in the MRM mode. During the heat stress period, pyruvic acid levels increased by 12% compared to the recovery period. This aligns with findings indicating enhanced glycolysis during heat stress in dairy cows, where pyruvate, a pivotal glycolytic intermediate, is converted into acetyl-CoA for the TCA cycle to meet energy demands under aerobic conditions (24,61). This metabolic shift supports increased energy production necessary to compensate for the physiological stress caused by elevated temperatures (62). Additionally, the levels of 3-hydroxybutyric acid (BHBA), a key ketone body, increased by more than 10% during heat stress, while citric acid levels declined. Elevated BHBA levels are a clear indicator of enhanced fatty acid oxidation and ketogenesis, mechanisms that contribute to the onset of heat-stress-induced ketosis (63). The decrease in citric acid, an important TCA cycle intermediate, suggests a shift away from carbohydrate metabolism, favoring ketone body production instead, as has been observed during ketosis (49,61). Increased levels of succinic and malic acids, both TCA cycle intermediates, further indicate an upregulation of the TCA cycle to sustain energy production during heat stress (64,65). The liver plays a central role in this metabolic shift, converting long-chain fatty acids (FFAs) like oleic and linoleic acids into acetyl-CoA through β-oxidation to fuel ketone body production demands (64),(66). Moreover, the levels of palmitic and stearic acids, which increased during heat stress, have been associated with the formation of ketone bodies, suggesting that fatty acids are being mobilized to address the energy deficit induced by heat stress (66,67). This mobilization of body fat highlights lipid catabolism as a key adaptive response to meet the energy demands imposed by heat stress. Additionally, the elevated serum levels of glucogenic amino acids (AAs) such as glutamine and phenylalanine during heat stress underscore their role in generating key intermediates like pyruvate and α-ketoglutaric acid, which feed into the TCA cycle to sustain energy production (66). Elevated creatinine levels, a marker of creatine phosphate breakdown, further reflect the increased utilization of creatine stores to meet the heightened ATP demand during heat stress (24). Elevated ornithine concentrations, a component of the urea cycle, have been observed under heat stress conditions (66). Furthermore, the increased levels of ketogenic amino acids like isoleucine and tyrosine during heat stress point toward their contribution to ketone body

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production, further supporting the metabolic shift towards ketosis (61). Meanwhile, the reduced availability of arginine, which is catabolized during immune responses, indicates its increased utilization in the body's response to heat stress, highlighting the interconnectedness of energy metabolism and immune activation (68). The metabolism of non-essential AAs such as glutamine and glutamic acid is interconnected owing to the ability of glutamine to undergo glutaminolysis (69). Glutamine transcends its role as a proteinogenic amino acid, functioning as a precursor for nucleotides (purines and pyrimidines), and directly supporting immune function through: nitric oxide/cytokine production, lymphocyte proliferation, fueling NADPH synthesis in macrophages for superoxide generation, and potentially serving as a metabolic substrate for immune cells (69–71). Branched-chain AAs (leucine, isoleucine, and valine) are essential for regulating energy homeostasis, nutritional metabolism, gut health, immune responses, and disease progression in humans and animals (72).

Additionally, heatmap analysis confirmed the clustering of the heat stress and recovery periods. The discriminatory metabolites were mainly AAs, implying that perturbations in AA metabolism were associated with heat stress. This study revealed distinct profiles of OAs), AAs, and FAs between the heat stress and recovery periods. Consequently, altered levels of OAs, AAs, and FAs likely contribute to the metabolic disruptions observed in dairy cows experiencing heat stress.

During the heat stress period, the concentrations of HSP27, HSP70, and HSP90 in the blood serum were higher than those in the recovery period. This increase can be attributed to the upregulation of transcriptionally active heat shock factors, which enhance the expression of HSPs. These HSPs play a role in promoting the refolding of misfolded proteins (73), thereby protecting cells from potential damage caused by hyperthermia during heat stress (74). Elevated levels of HSPs are a rapid protective mechanism employed by the body to counteract heat stress and maintain homeostasis (75).

Furthermore, heat stress may induce hypoglycemia via a confluence of factors: diminished dietary intake (reduced dry matter consumption), heightened thermoregulatory demands, and a heat-mediated suppression of gluconeogenesis, representing an endocrine adaptation to maintain homeostasis (76). Ronchi et al.(13) demonstrated that the liver activity in cows is diminished under hot conditions, negatively

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affecting gluconeogenesis and leading to decreased blood glucose levels. Additionally, stress can impair liver function (77), as indicated by increased levels of liver damage markers such as glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase (78) under high THI conditions (79). Ruminants typically experience a postprandial decrease in glucose (76), and lower blood glucose levels during bitter periods may be a result of higher feed intake during night in these seasons. The concentration of ketone bodies in the blood serum significantly increases during heat stress, which can be attributed to adaptive responses arising from diminished energy intake and an altered net energy balance (5).The process of body fat mobililization can instigate the generation of ketone bodies, serving as vital energy sources or contributing to the synthesis of milk fat (5,80,81). BUN levels were significantly lower during the heat stress period as compared to the subsequent recovery period. This contradicts the results of previous studies that reported an increase in BUN during heat stress, likely due to the increased utilization of AAs as an energy source (13,76). However, there is inconsistency in the effect of heat stress on serum BUN concentration, as some studies have reported an increase (82) whereas others have reported no change (13). The absorptive function of the rumen epithelium may decrease during heat stress, resulting in reduced BUN reabsorption and accumulation in blood(83). Cows and heifers subjected to heat stress (13,84) have shown elevated levels of plasma urea nitrogen. The observed rise likely stems from either rumen-related inefficiencies in ammonia utilization or the deamination of skeletal muscle-derived amino acids within the liver (77). Elevated ambient temperature (85), have been associated with decreased phosphorus and calcium levels, potentially linked to diminished mineral retention in response to potassium loss through increased sweating (79). In contrast, heat stress has been found to result in heightened blood magnesium levels, which can be attributed to increased utilization of magnesium by lipolytic enzymes and a reduction intransportation through the rumen (86). The total protein concentration in the blood serum was significantly higher during heat stress than during the recovery period. This contradicts previous studies (87,88,89) that reported a decrease in total protein concentration during heat exposure. However, high total protein levels during heat stress may be attributed to maternal protein requirements for milk production and immunoglobulin synthesis (90), as heat stress and recovery periods occur at different lactation stages. Changes in the activity of AST, an enzyme involved in AA and carbohydrate metabolism (91), in blood can indicate increased cellular activity or

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damage to the cell structure (92). AST concentration in dairy cows was significantly higher during the early lactation stage than during subsequent periods. Increased serum AST levels indicate liver damage (92,93). Additionally, significantly higher total bilirubin levels were observed during heat stress, suggesting decreased liver excretory capacity (94). Liver function changes during heat stress, potentially affecting health and metabolic acclimation (84). Elevated liver lipidosis resulting from increased energy demands for thermoregulation and decreased feed intake in heat-stressed dairy cows can adversely affect liver function, leading to decreased albumin secretion and reduced liver enzyme activity (13). Abeni et al (76). partially confirmed a reduction in liver activity during heat stress. Reduced liver activity, as observed by According to Ronchi et al. (13), decreased liver activity may account for the observed reduction in blood cholesterol levels observed during hot periods (76). The decrease in plasma cholesterol and triglyceride levels could be attributed to increased lipid utilization by peripheral tissues during heat stress (76).

Cincović et al. (94) reported that heat-stressed cows exhibited lower RBC, HCT, and HGB, which they attributed to the hemodilution effect due to increased water consumption for evaporative cooling. Our study observed a similar trend, with a significant decrease in RBC and HGB levels in heat-stressed cows compared to those in thermoneutral conditions. This finding reinforces the hypothesis that hemodilution may play a key role in altering blood parameters during heat stress, as proposed by Cincović et al. (94). In contrast, we did not observe the increase in RBC, HCT, and HGB as suggested by Cincović et al. (94) in response to high temperatures. This discrepancy could be due to differences in study design, duration of heat exposure, or genetic variations among the cattle breeds studied. Our results align with the hypothesis that the hemodilution effect is a predominant response to heat stress, at least under the specific environmental conditions and cattle breed we investigated. Cincović et al. (94) found that heat-stressed cows exhibited lower RBC, HCT (erythrocyte ratio), and HGB compared to cows in a thermoneutral condition. This could be attributed to the hemodilution effect resulting from increased water consumption for evaporative cooling. Conversely, cows exposed to high temperatures show increased RBC counts, HCT, and HGB, indicating increased hemoconcentration (94). Regarding WBC levels, our findings are consistent with those of Morar and Hutu (85), who reported increased WBC in heat-stressed dairy cattle. In our study, WBC counts were also elevated under heat stress, likely as a result of the animal's immune response to

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thermal stress. However, we observed a more nuanced immune response than some of the earlier studies. While Morar and Hutu reported an increase in neutrophils, lymphocytes, and monocytes, our results showed elevated neutrophils but no significant changes in lymphocyte or monocyte counts. This could indicate a more selective immune response in our study population, possibly related to differences in the duration or severity of heat stress, as well as environmental and management factors. Contrary to the findings of Mazzullo et al. (95), who reported elevated lymphocyte counts and depressed neutrophil and monocyte levels, we observed an increase in neutrophils without significant changes in lymphocytes or monocytes. This discrepancy suggests variability in how the immune system of dairy cows responds to heat stress across different studies. The differences in immune response could be due to varying levels of environmental heat stress or differing genetic and physiological characteristics of the animals. Our study did not find significant changes in eosinophil counts, although some studies, such as those by Majkic et al. (96), have reported elevated eosinophil counts during heat stress. This difference may be due to variations in the degree of heat exposure, as eosinophils are particularly sensitive to the intensity and duration of thermal stress. Additionally, eosinophil response may be influenced by factors such as parasitic infections or allergic conditions, which were not a focus of our study. Moreover, the observed inconsistencies in immune responses across studies (97–99) were also evident in our findings. Similar to reports by Cincovic et al. (100), we found that elevated ambient temperatures over an extended period resulted in a reduced lymphocyte viability, as indicated by a diminished lymphocyte response to mitogenic stimuli. This further supports the notion that chronic heat stress has a detrimental effect on immune cell function. However, our study also points to a complex interplay between different immune cell types during heat stress, underscoring the need for further research to elucidate the mechanisms driving these varied responses.

Previous studies (32,101) suggest that heat stress can disrupt rumen homeostasis by shifting the balance of acid-producing and acid-utilizing bacteria, potentially lowering ruminal pH. Specifically, heat stress tends to increase lactic acid producers like *Lactobacillus* while reducing fiber-degrading bacteria such as *Ruminococcus* (101). However, in the current study, no significant difference in pH was observed between heat and recovery periods. This could be attributed to the resilience of ruminal buffering mechanisms, which may stabilize pH even as microbial shifts occur (102,103). The normalization of feed

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intake and rumination during recovery likely restored salivary buffering capacity, counteracting any potential pH decline (Larsen et al., 2021). Thus, while microbial alterations are evident, they did not significantly impact ruminal pH in this study. In lactating goats, heat stress has been shown to significantly increase ruminal NH_3-N (83). Similar findings have been observed in cattle subjected to increased roughage levels during heat stress periods (83,104). However, prior research on the impact of heat stress on ruminal NH_3-N levels shows conflicting results . Notably, Cai et al. (83) reported a significant decrease in NH_3-N concentration under heat stress conditions, indicating that the challenges posed by heat stress can affect rumen fermentation, digestion, and the metabolism of dietary protein and nitrogen-containing compounds. Though present results show numerically higher acetate and butyrate levels during heat stress, prior research suggests a potential decrease in acetate proportion and increase in butyrate proportion within the rumen under such conditions (83,105). Researchers have proposed that heat stress augments water demand and content in the rumen, leading to alterations in VFA concentrations (83,104–106). During heat stress, peripheral blood flow increases for thermoregulation, leading to a concomitant reduction in splanchnic perfusion and potentially diminished VFA uptake . This translates to a rise in total rumen volatile fatty acid (VFA) concentration, followed by a corresponding decline in rumen pH (83). Consistent with the present findings, Uyeno et al. (107) observed that heat stress significantly increased concentrate intake and consequently increased propionate production. Therefore, decreased feed intake, altered diet preferences, and shifts in rumen microbiota abundance or activity are critical factors that influence propionate production (102,105–107).

Despite experiencing heat stress, the cows in this study exhibited no statistically significant changes in milk yield, lipid content, or protein content. A numerical decline in milk yield was observed during the recovery phase, warranting further investigation. This finding is significant because it implies that the shedding of mammary epithelial cells during and after heat stress may increase the somatic cell count, compensating for the loss of milk production (108). Although this study did not directly examine feed intake, it is plausible that decreased feed intake played a role in the observed milk loss (109–111). Furthermore, the decline in milk fat and protein content may be driven by the specific downregulation of thermoregulatory activity in mammary protein synthesis and changes in AA and glucose transport

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(112,113). High ambient temperatures and humidity impede evaporative cooling mechanisms in cattle, leading to heat stress and subsequent physiological alterations that compromise milk yield and composition (114).

Established research on ruminant lactation demonstrates a clear association between lactation stage and both milk yield and composition (115,116). Kuczyńska et al. (117) found that milk yield was considerably higher during the early stages of lactation (< 100 DIM) than during mid-lactation (101–200 DIM), regardless of parity, with an increase of > 10%. Conversely, heat stress resulted in a reduction in SNF by approximately 10% compared to the recovery period. This finding is consistent with the results reported by Garcia et al. (118), where SNF decreased across all groups of heat-stressed animals. The decrease in SNF during the heat stress period has been primarily attributed to a reduction in milk protein (21), although no significant reduction in milk protein was observed in the present study. Furthermore, heat stress leads to a decrease in milk urea nitrogen, possibly because of disturbances in urea circulation within the bloodstream (119). Roberts et al. (120) noted that milk urea levels were the highest after 90 days of lactation, in contrast to the very early lactation stage observed during the heat stress period in the present study. Additionally, excess urea in milk is associated with elevated nitrogen excretion through feces and urine, which signifies energy expenditure for the animal (121), a phenomenon observed during the recovery period in the present study.

In the present study, heat stress during the early stages of lactation significantly altered the rumen fermentation characteristics of Holstein dairy cows, specifically by lowering propionate and total VFA concentrations during the recovery period. This observation aligns with previous studies (31,122) that demonstrate how heat stress affects the rumen microbial environment, leading to shifts in the production of SCFAs and VFAs. Rumen fermentation is largely dependent on the activity of the microbial population, which in turn is highly sensitive to temperature fluctuations (123). During heat stress, microbial populations that specialize in fermenting carbohydrates into VFAs may be suppressed, particularly those responsible for propionate production, such as *Prevotella spp*. and *Bacteroides spp.* (124). The decline in propionate production, as observed in this study, may result from the inhibition of these microbial populations under heat stress conditions. Propionate is a key gluconeogenic precursor, and a reduction in its production could

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contribute to the observed lower serum glucose levels . This suggests a direct link between microbial shifts and metabolic changes in the host. We observed a decline in *Gilliamella bombicola, Gilliamella bombi, Lentimicrobium saccharophilum, Paludibacter propionicigenes, Treponema bryantii, Prevotella scopos, Anaerocolumna cellulosilytica, Treponema saccharophilum, Vallitalea pronyensis,* and *Prevotella marshii* during heat stress. Additionally, the overall reduction in total VFAs during the recovery period could indicate a lag in the re-establishment of normal microbial function and fermentation activity following heat stress. Research suggests that heat stress not only alters microbial community structure but also reduces microbial diversity and disrupts the balance of key fermentative pathways (17). This disruption can impair the efficient breakdown of carbohydrates and subsequent VFA production, further contributing to the metabolic imbalances observed during heat stress. The elevated blood ketone levels in our study may be indicative of increased lipolysis and ketogenesis, potentially a compensatory mechanism in response to reduced glucose availability due to lower propionate production. Furthermore, the increase in total protein, AST, and total bilirubin concentrations during heat stress suggests hepatic stress and altered protein metabolism, which may also be linked to the reduced availability of VFAs as an energy source.

The study reveals that heat stress significantly impacts the rumen microbial composition, metabolism, and immune function of dairy cows, despite stable microbial diversity. The microbial shifts, particularly in the genera Gilliamella, Lentimicrobium, and Treponema, affect crucial fermentation processes, including the production of volatile fatty acids (VFAs) such as propionate, which supports energy metabolism. Heat stress induces metabolic adaptations characterized by increased lipolysis, ketogenesis, and changes in amino acid metabolism, contributing to an elevated production of ketone bodies. Additionally, the liver exhibits signs of stress, as indicated by higher levels of heat shock proteins (HSPs) and enzymes, reflecting an upregulation of protective mechanisms. These alterations underline the resilience of the rumen and liver in maintaining function under heat stress, although with significant shifts in energy metabolism and immune responses, requiring further investigation into long-term impacts on health and productivity.

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References

1. Deser C, Phillips A, Bourdette V, Teng H. Uncertainty in climate change projections: The role of internal variability. Clim Dyn. 2012;38:527–46. https://doi.org/10.1007/s00382-010-0977-x

2. Buse A, Dury SJ, Woodburn RJW, Perrins CM, Good JEG. Effects of elevated temperature on multi-species interactions: The case of Pedunculate Oak, Winter Moth and Tits. Funct Ecol. 1999;13:74–82. https://doi.org/10.1046/j.1365-2435.1999.00010.x

3. Sepulveda J, Moeller AH. The effects of temperature on animal gut microbiomes. Front Microbiol. 2020;11(March):1–9. https://doi.org/10.3389/fmicb.2020.00384

4. Cartwright SL, Schmied J, Karrow N, Mallard BA. Impact of heat stress on dairy cattle and selection strategies for thermotolerance: a review. Front Vet Sci. 2023;10(June):1–13. https://doi.org/10.3389/fvets.2023.1198697

5. Tian H, Wang W, Zheng N, Cheng J, Li S, Zhang Y, et al. Identification of diagnostic biomarkers and metabolic pathway shifts of heat-stressed lactating dairy cows. J Proteomics. 2015; 1 (July)125:17-28. doi: 10.1016/j.jprot.2015.04.014

6. Itoh F, Obara Y, Rose MT, Fuse H, Hashimoto H. Insulin and Glucagon Secretion in Lactating Cows during Heat Exposure. J Anim Sci. 1998;76(8):2182–9. doi: 10.2527/1998.7682182x

7. Shwartz G, Rhoads ML, Vanbaale MJ, Rhoads RP, Baumgard LH. Effects of a supplemental yeast culture on heat-stressed lactating Holstein cows. J Dairy Sci. 2009;92:935–42. doi: 10.3168/jds.2008-1496

8. Sammad A, Wang YJ, Umer S, Lirong H, Khan I, Khan A, et al. Nutritional physiology and biochemistry of dairy cattle under the influence of heat stress: Consequences and opportunities. Animals. 2020;10(5). https://doi.org/10.3390/ani10050793

9. Yadav B, Singh G, Verma AK, Dutta N, Sejian V. Impact of heat stress on rumen functions. Vet World. 2013;6(12):992–6. Available from: www.veterinaryworld.org/Vol.6/Dec-2013/10.pdf

10. Huang L, Xu Y. Effective reduction of antinutritional factors in soybean meal by acetic acid-catalyzed processing. J Food Process Preserv. 2018;42(11). https://doi.org/10.1111/jfpp.13775

11. Abilay TA, Johnson HD, Madan M. Influence of Environmental Heat on Peripheral Plasma Progesterone and Cortisol During the Bovine Estrous Cycle. J Dairy Sci [Internet]. 1975;58(12):1836–40. Available from: http://dx.doi.org/10.3168/jds.S0022-0302(75)84795-3

12. Nazifi S, Gheisari HR, Poorabbas H. The influences of thermal stress on serum biochemical

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parameters of dromedary camels and their correlation with thyroid activity. Comp Clin Path. 1999;9(1):49–53. https://doi.org/10.1007/PL00010007

13. Ronchi B, Stradaioli G, Supplizi AV, Bernabucci U, Lacetera N, Accorsi PA, et al. Ronchi-Heat Stress and Feed Restriction and Reproduction. 2001;68:231–41. https://doi.org/10.1016/S0301-6226(00)00232-3

14. Asri Rezaei S, Dalir-Naghadeh B. Evaluation of antioxidant status and oxidative stress in cattle naturally infected with Theileria annulata. Vet Parasitol. 2006;142(1–2):179–86. https://doi.org/10.1016/j.vetpar.2006.05.033.

15. Bishop-Williams KE, Berke O, Pearl DL, Hand K, Kelton DF. Heat stress related dairy cow mortality during heat waves and control periods in rural Southern Ontario from 2010-2012. BMC Vet Res. 2015;11(1):1–10. https://doi.org/10.1186/s12917-015-0607-2

16. De Rensis F, Garcia-Ispierto I, López-Gatius F. Seasonal heat stress: Clinical implications and hormone treatments for the fertility of dairy cows. Theriogenology. 2015;84(5):659–66. https://doi.org/10.1016/j.theriogenology.2015.04.021.

17. Koch F, Lamp O, Eslamizad M, Weitzel J, Kuhla B. Metabolic Response to heat stress in late-pregnant and early lactation dairy cows: Implications to liver-muscle crosstalk. PLoS One. 2016;11(8):1–19. doi: 10.1371/journal.pone.0160912

18. Hailemariam D, Mandal R, Saleem F, Dunn SM, Wishart DS, Ametaj BN. Identification of predictive biomarkers of disease state in transition dairy cows. J Dairy Sci. 2014;97(5):2680–93. https://doi.org/10.3168/jds.2013-6803.

19. Dechow CD, Goodling RC. Mortality, culling by sixty days in milk, and production profiles in high- and low-survival Pennsylvania herds. J Dairy Sci. 2008;91(12):4630–9. doi: 10.3168/jds.2008-1337

20. Vitali A, Segnalini M, Bertocchi L, Bernabucci U, Nardone A, Lacetera N. Seasonal pattern of mortality and relationships between mortality and temperature-humidity index in dairy cows. J Dairy Sci. 2009;92:3781–90. doi: 10.3168/jds.2009-2127

21. Kadzere CT, Murphy MR, Silanikove N, Maltz E. Heat stress in lactating dairy cows: A review. Livest Prod Sci. 2002;77:59–91. https://doi.org/10.1016/S0301-6226(01)00330-X.

22. Polsky L, von Keyserlingk MAG. Invited review: Effects of heat stress on dairy cattle welfare. J Dairy Sci [Internet]. 2017;100(11):8645–57. Available from: http://dx.doi.org/10.3168/jds.2017-12651

23. West JW. Effects of heat-stress on production in dairy cattle. J Dairy Sci [Internet]. 2003;86(6):2131–44. Available from: http://dx.doi.org/10.3168/jds.S0022-0302(03)73803-X

ACCEPTED

24. Baumgard LH, Rhoads RP. Effects of heat stress on postabsorptive metabolism and energetics. Annu Rev Anim Biosci. 2013;1:311–37. doi: 10.1146/annurev-animal-031412-103644

25. O'Brien MD, Rhoads RP, Sanders SR, Duff GC, Baumgard LH. Metabolic adaptations to heat stress in growing cattle. Domest Anim Endocrinol. 2010;38(2):86–94. doi: 10.1016/j.domaniend.2009.08.005.

26. McCann JC, Wickersham TA, Loor JJ. High-throughput methods redefine the rumen microbiome and its relationship with nutrition and metabolism. Bioinform Biol Insights. 2014;8:109–25. 2014;8:109–25. doi: 10.4137/BBI.S15389.

27. Golder HM, Denman SE, McSweeney C, Wales WJ, Auldist MJ, Wright MM, et al. Effects of partial mixed rations and supplement amounts on milk production and composition, ruminal fermentation, bacterial communities, and ruminal acidosis. J Dairy Sci. 2014;97(9):5763–85. https://doi.org/10.3168/jds.2014-8049.

28. Jami E, Israel A, Kotser A, Mizrahi I. Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 2013;7(6):1069–79. https://doi.org/10.1038/ismej.2013.2

29. Prendiville R, Lewis E, Pierce KM, Buckley F. Comparative grazing behavior of lactating Holstein-Friesian, Jersey, and Jersey × Holstein-Friesian dairy cows and its association with intake capacity and production efficiency. J Dairy Sci. 2010;93(2):764–74. https://doi.org/10.3168/jds.2009-2659.

30. Weimer PJ, Stevenson DM, Mantovani HC, Man SLC. Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents1. J Dairy Sci. 2010;93(12):5902–12. doi: 10.3168/jds.2010-3500

31. Feng L, Zhang Y, Liu W, Du D, Jiang W, Wang Z, et al. in heat-stressed dairy cows at different growth stages. Microbiol Spectr. 2023;11(6). https://doi.org/10.1128/spectrum.03312-23

32. Kim DH, Kim MH, Kim SB, Son JK, Lee JH, Joo SS, et al. Differential dynamics of the ruminal microbiome of jersey cows in a heat stress environment. Animals. 2020;10(7):1–19. https://doi.org/10.3390/ani10071127

33. Eslamizad M, Albrecht D, Kuhla B. The effect of chronic, mild heat stress on metabolic changes of nutrition and adaptations in rumen papillae of lactating dairy cows. J Dairy Sci [Internet]. 2020 Sep;103(9):8601–14. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022030220304896

34. Kim SH, Ramos SC, Valencia RA, Cho Y Il, Lee SS. Heat Stress: Effects on Rumen Microbes and Host Physiology, and Strategies to Alleviate the Negative Impacts on Lactating Dairy Cows. Front Microbiol [Internet]. 2022 Feb 28;13(February):1–23. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2022.804562/full

ACCEPTED

35. Nasrollahi SM, Zali A, Ghorbani GR, Khani M, Maktabi H, Beauchemin KA. Effects of increasing diet fermentability on intake, digestion, rumen fermentation, blood metabolites and milk production of heat-stressed dairy cows. Animal [Internet]. 2019;13(11):2527–35. Available from: http://dx.doi.org/10.1017/S1751731119001113

36. Guo Z, Gao S, Ding J, He J, Ma L, Bu D. Effects of Heat Stress on the Ruminal Epithelial Barrier of Dairy Cows Revealed by Micromorphological Observation and Transcriptomic Analysis. Front Genet [Internet]. 2022 Jan 13;12(January):1–9. Available from: https://www.frontiersin.org/articles/10.3389/fgene.2021.768209/full

37. Illumina. 16S Metagenomic Sequencing Library [Internet]. Illumina.com. 2013. p. 1–28. Available from: http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

38. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 2011;17(1):10–2. https://doi.org/10.14806/ej.17.1.200

39. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581–3. https://doi.org/10.1038/nmeth.3869

40. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods [Internet]. 2010 May 11;7(5):335–6. Available from: https://www.nature.com/articles/nmeth.f.303

41. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture and applications. BMC Bioinformatics [Internet]. 2009 Dec 15;10(1):421. Available from: https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-10-421

42. Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res [Internet]. 2017 Jul 3;45(W1):W180–8. Available from: https://academic.oup.com/nar/article-lookup/doi/10.1093/nar/gkx295

43. Chong J, Liu P, Zhou G, Xia J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc [Internet]. 2020 Mar 15;15(3):799–821. Available from: http://dx.doi.org/10.1038/s41596-019-0264-1

44. Miguel MA, Kim SH, Lee SS, Cho Y Il. Impact of soil microbes and oxygen availability on bacterial community structure of decomposing poultry carcasses. Animals. 2021;11(10).

45. Kim Y, Kim SH, Oh SJ, Lee HS, Ji M, Choi S, et al. Metabolomic analysis of organic acids, amino acids, and fatty acids in plasma of Hanwoo beef on a high-protein diet. Metabolomics. 2020;16(10).

ACCEPTED

https://doi.org/10.1007/s11306-020-01737-4

46. Lee HS, Seo C, Kim YA, Park M, Choi B, Ji M, et al. Metabolomic study of polyamines in rat urine following intraperitoneal injection of γ-hydroxybutyric acid. Metabolomics. 2019;15(4). doi: 10.1007/s11306-019-1517-2.

47. Seo C, Kim SH, Lee HS, Ji M, Min J, Son YJ, et al. Metabolomic study on bleomycin and polyhexamethylene guanidine phosphate-induced pulmonary fibrosis mice models. Metabolomics. 2019;15(8). doi: 10.1007/s11306-019-1574-6

48. Clemmons BA, Powers JB, Campagna SR, Seay TB, Embree MM, Myer PR. Rumen fluid metabolomics of beef steers differing in feed efficiency. Metabolomics. 2020;16(2):1–9. doi: 10.1007/s11306-020-1643-x

49. Wang Y, Gao Y, Xia C, Zhang H, Qian W, Cao Y. Pathway analysis of plasma different metabolites for dairy cow ketosis. Ital J Anim Sci. 2016;15(3):545–51. https://doi.org/10.1080/1828051X.2016.1180643

50. Chaney AL, Marbach EP. Modified reagents for determination of urea and ammonia. Clin Chem. 1962;8(2):130–2. https://doi.org/10.1093/clinchem/8.2.130

51. Miguel M, Mamuad L, Ramos S, Ku MJ, Jeong CD, Kim SH, et al. Effects of using different roughages in the TMR inoculated with or without coculture of *Lactobacillus acidophilus* and *Bacillus subtilis* on *in vitro* rumen fermentation and microbial population. Asian-Australasian J Anim Sci. 2020;34(4):642–51. doi: 10.5713/ajas.20.0386.

52. Wang J, Li J, Wang F, Xiao J, Wang Y, Yang H, et al. Heat stress on calves and heifers: A review. J Anim Sci Biotechnol. 2020;11(1):1–8. https://doi.org/10.1186/s40104-020-00485-8

53. Weimer PJ. Redundancy, resilience, and host specificity of the ruminal microbiota: Implications for engineering improved ruminal fermentations. Front Microbiol. 2015;6(APR):1–16. https://doi.org/10.3389/fmicb.2015.00296

54. Xie X, Yang C, Guan LL, Wang J, Xue M, Liu JX. Persistence of cellulolytic bacteria fibrobacter and treponema after short-term corn stover-based dietary intervention reveals the potential to improve rumen fibrolytic function. Front Microbiol. 2018;9(JUN):1–15. doi: 10.3389/fmicb.2018.01363

55. Louca S, Polz MF, Mazel F, Albright MBN, Huber JA, O'Connor MI, et al. Function and functional redundancy in microbial systems. Nat Ecol Evol [Internet]. 2018;2(6):936–43. Available from: http://dx.doi.org/10.1038/s41559-018-0519-1

56. Islam M, Kim SH, Son AR, Ramos SC, Jeong CD, Yu Z, et al. Seasonal influence on rumen microbiota, rumen fermentation and enteric methane emissions of holstein and jersey steers under the

ACCEPTED

same total mixed ration. Animals. 2021;11(4). https://doi.org/10.3390/ani11041184

57. Yadav B, Yadav P, Kumar M, Vasvani S, Anand M, Kumar A, et al. Effect of Heat Stress on Rumen Microbial Diversity and Fermentation Pattern in Buffalo. Adv Gut Microbiome Res. 2022;2022:1–14. https://doi.org/10.1155/2022/1248398

58. Park T, Ma L, Gao S, Bu D, Yu Z. Heat stress impacts the multi-domain ruminal microbiota and some of the functional features independent of its effect on feed intake in lactating dairy cows. J Anim Sci Biotechnol. 2022;13(1):1–15. https://doi.org/10.1186/s40104-022-00717-z

59. Fu Y, He Y, Xiang K, Zhao C, He Z, Qiu M, et al. The Role of Rumen Microbiota and Its Metabolites in Subacute Ruminal Acidosis (SARA)-Induced Inflammatory Diseases of Ruminants. Microorganisms [Internet]. 2022 Jul 25;10(8):1495. Available from: https://www.mdpi.com/2076-2607/10/8/1495

60. Sun Z, Aschalew ND, Cheng L, Xia Y, Zhang L, Yin G, et al. Dietary 5-hydroxytryptophan improves sheep growth performance by enhancing ruminal functions, antioxidant capacity, and tryptophan metabolism: in vitro and in vivo studies. Front Immunol. 2024;15(May):1–20. https://doi.org/10.3389/fimmu.2024.1398310

61. Monteiro APA, Tao S, Thompson IMT, Dahl GE. In utero heat stress decreases calf survival and performance through the first lactation. J Dairy Sci [Internet]. 2016;99(10):8443–50. Available from: http://dx.doi.org/10.3168/jds.2016-11072

62. DiGiacomo K, Simpson S, Leury BJ, Dunshea FR. Dietary Betaine Impacts Metabolic Responses to Moderate Heat Exposure in Sheep. Animals. 2023;13(10). doi: 10.3390/ani13101691

63. Wheelock JB, Rhoads RP, VanBaale MJ, Sanders SR, Baumgard LH. Effects of heat stress on energetic metabolism in lactating Holstein cows. J Dairy Sci [Internet]. 2010;93(2):644–55. Available from: http://dx.doi.org/10.3168/jds.2009-2295

64. Hou Y, Wang X, Ping J, Lei Z, Gao Y, Ma Z, et al. Metabonomics Approach to Assessing the Modulatory Effects of Kisspeptin-10 on Liver Injury Induced by Heat Stress in Rats. Sci Rep. 2017;7(1):1–10. doi: 10.1038/s41598-017-06017-1

65. Shi Y, Wang D. Implication of metabolomic profiles to wide thermoneutral zone in Mongolian gerbils (Meriones unguiculatus). Integr Zool. 2016;11(4):282–94. doi: 10.1111/1749-4877.12179

66. Liao Y, Hu R, Wang Z, Peng Q, Dong X, Zhang X, et al. Metabolomics profiling of serum and urine in three beef cattle breeds revealed different levels of tolerance to heat stress. J. Agric. Food Chem. 2018. 6926–6935. 66: 6926–6935. https://doi.org/10.1021/acs.jafc.8b01794

67. Collier RJ, Beede DK, Thatcher WW, Israel LA, Wilcox CJ. Influences of Environment and Its

ACCEPTED

Modification on Dairy Animal Health and Production. J Dairy Sci [Internet]. 1982;65(11):2213–27. Available from: http://dx.doi.org/10.3168/jds.S0022-0302(82)82484-3

68. Flynn NE, Meininger CJ, Haynes TE, Wu G. The metabolic basis of arginine nutrition and pharmacotherapy. Biomed Pharmacother. 2002;56(9):427–38. doi: 10.1016/s0753-3322(02)00273-1

69. Newsholme P, Curi R, Pithon Curi TC, Murphy CJ, Garcia C, Pires De Melo M. Glutamine metabolism by lymphocytes, macrophages, and neutrophils: Its importance in health and disease. J Nutr Biochem. 1999;10(6):316–24. doi: 10.1016/s0955-2863(99)00022-4.

70. Yassad A, Lavoinne A, Bion A, Daveau M, Husson A. Glutamine accelerates interleukin-6 production by rat peritoneal macrophages in culture. FEBS Lett. 1997;413(1):81–4. https://doi.org/10.1016/S0014-5793(97)00881-8.

71. Furukawa S, Saito H, Inoue T, Matsuda T, Fukatsu K, Han I, et al. Supplemental glutamine augments phagocytosis and reactive oxygen intermediate production by neutrophils and monocytes from postoperative patients in vitro. Nutrition. 2000;16(5):323–9. doi: 10.1016/s0899-9007(00)00228-8

72. Coleman DN, Lopreiato V, Alharthi A, Loor JJ. Amino acids and the regulation of oxidative stress and immune function in dairy cattle. J Anim Sci. 2020;98(October 2019):S175–93. https://doi.org/10.1093/jas/skaa138

73. Li G, Ali IS, Currie RW. Insulin induces myocardial protection and Hsp70 localization to plasma membranes in rat hearts. Am J Physiol - Hear Circ Physiol. 2006;291(4). doi: 10.1152/ajpheart.00201.2006

74. Lee WC, Wen HC, Chang CP, Chen MY, Lin MT. Heat shock protein 72 overexpression protects against hyperthermia, circulatory shock, and cerebral ischemia during heatstroke. J Appl Physiol. 2006;100(6):2073–82. doi: 10.1152/japplphysiol.01433

75. Min L, Cheng J bo, Shi B lu, Yang H jian, Zheng N, Wang J qi. Effects of heat stress on serum insulin, adipokines, AMP-activated protein kinase, and heat shock signal molecules in dairy cows. J Zhejiang Univ Sci B. 2015;16(6):541–8. doi: 10.1631/jzus.B1400341

76. Abeni F, Calamari L, Stefanini L. Metabolic conditions of lactating Friesian cows during the hot season in the Po valley. 1. Blood indicators of heat stress. Int J Biometeorol. 2007; doi: 10.1007/s00484-007-0098-3

77. Bernabucci U, Lacetera N, Baumgard LH, Rhoads RP, Ronchi B, Nardone A. Metabolic and hormonal acclimation to heat stress in domesticated ruminants. Animal. 2010;4(7):1167–83. https://doi.org/10.1017/S175173111000090X.

78. Panteghini M. Aspartate aminotransferase isoenzymes. Clin Biochem. 1990; 23(4):311-9. doi:

ACCEPTED

10.1016/0009-9120(90)80062-n

79. Kang HJ, Piao MY, Lee IK, Kim HJ, Gu MJ, Yun CH, et al. Effects of ambient temperature and dietary glycerol addition on growth performance, blood parameters and immune cell populations of Korean cattle steers. Asian-Australasian J Anim Sci. 2017; 30(4):505-513. doi: 10.5713/ajas.16.0474

80. Drackley JK, Veenhuizen JJ, Richard MJ, Young JW. Metabolic Changes in Blood and Liver of Dairy Cows During Either Feed Restriction or Administration of 1,3-Butanediol. J Dairy Sci. 1991;74(12):4254–64. https://doi.org/10.3168/jds.S0022-0302(91)78620-7

81. Peterson SE, Rezamand P, Williams JE, Price W, Chahine M, McGuire MA. Effects of dietary betaine on milk yield and milk composition of mid-lactation Holstein dairy cows. J Dairy Sci. 2012; 11 (95):6557-6562. https://doi.org/10.3168/jds.2011-4808.

82. Habeeb A., Fatma FIT, Osman S. Detection of heat adaptability using heat shock proteins and some hormones in Egyptian Buffalo calves. Egypt J Appl Sci. 2007; 2007:28-53.

83. Cai L, Yu J, Hartanto R, Zhang J, Yang A, Qi D. Effects of heat challenge on growth performance, ruminal, blood and physiological parameters of Chinese crossbred goats. Small Rumin Res. 2019; 174:125-130. https://doi.org/10.1016/j.smallrumres.2019.02.021.

84. Basiricò L, Bernabucci U, Morera P, Lacetera N, Nardone A. Gene expression and protein secretion of apolipoprotein B100 (ApoB100) in transition dairy cows under hot or thermoneutral environments. Ital J Anim Sci. 2009; 8: 492-594. https://doi.org/10.4081/ijas.2009.s2.592

85. Kume S, Kurihara M, Takahashi S, Shibata M, Aii T. Effect of hot environmental temperature on major mineral balance in dry cows. Nihon Chikusan Gakkaiho. 1986; 58 (9):764:770.

86. Calamari L, Abeni F, Calegari F, Stefanini L. Metabolic conditions of lactating Friesian cows during the hot season in the Po valley. 2. Blood minerals and acid-base chemistry. Int J Biometeorol. 2007; 52(2):87-96. doi: 10.1007/s00484-007-0098-3

87. Arieli A, Adin G, Bruckental I. The effect of protein intake on performance of cows in hot environmental temperatures. J Dairy Sci. 2004;87(3):620–9. https://doi.org/10.3168/jds.S0022-0302(04)73204-X.

88. Marai IFM, El-Darawany AA, Fadiel A, Abdel-Hafez MAM. Physiological traits as affected by heat stress in sheep-A review. Small Ruminant Research. 2007. https://doi.org/10.1016/j.smallrumres.2006.10.003

89. Marai IFM, Haeeb AAM. Buffalo's biological functions as affected by heat stress - A review. Livestock Science. 2010. https://doi.org/10.1016/j.livsci.2009.08.001. ci.2009.08.001

ACCEPTED

90. Piccione G, Messina V, Marafioti S, Casella S, Giannetto C, Fazio F. Changes of some haematochemical parameters in dairy cows during late gestation, post partum, lactation and dry periods. Vet ir Zootech. 2012;58(80):59–64.

91. Filipejová T, Kovacik J. Evaluation of selected biochemical parameters in blood plasma, urine and milk of dairy cows during the lactation period. Slovak J Anim Sci. 2009; 1:8-12.

92. Milinković-Tur S, Perić V, Stojević Z, Zdelar-Tuk M, Piršljin J. Concentrations of total proteins and albumins, and AST, ALT and GGT activities in the blood plasma of mares during pregnancy and early lactation. Vet Arh. 2005; 75 (3), 195-202.

93. Zimmerman HJ, Dujovne CA, Levy R. The correlation of serum levels of two transaminases with tissue levels in six vertebrate species. Comp Biochem Physiol. 1968; 3 (25):1081-1089. https://doi.org/10.1016/0010-406X(68)90593-8.

94. Cincović MR, Belić B, Toholj B, Potkonjak A, Stevančević M, Lako B, et al. Metabolic acclimation to heat stress in farm housed Holstein cows with different body condition scores. African J Biotechnol. 2011; 10(50):10293–303. doi: 10.5897/AJB11.847

95. Mazzullo G, Rifici C, Cammarata F, Caccamo G, Rizzo M, Piccione G. Effect of different environmental conditions on some haematological parameters in cow. Ann Anim Sci. 2014; 4 (2014) 947–954. doi: 10.2478/aoas-2014-0049

96. Majkić M, Cincović MR, Belić B, Plavša N, Hristov S, Stanković B, et al. Variations in milk production based on the temperature-humidity index and blood metabolic parameters in cows during exposure to heat stress. Anim Sci Pap Reports. 2019;36(4):359–69. Available at: http://aspace.agrif.bg.ac.rs/handle/123456789/5128

97. Kamwanja LA, Chase CC, Gutierrez JA, Guerriero V, Olson TA, Hammond AC, et al. Responses of bovine lymphocytes to heat shock as modified by breed and antioxidant status. J Anim Sci. 1994; 72(2):438-44. doi: 10.2527/1994.722438x

98. Soper FF, Muscoplat CC, Johnson DW. In vitro stimulation of bovine peripheral blood lymphocytes: analysis of variation of lymphocyte blastogenic response in normal dairy cattle. Am J Vet Res. 1978; 39(6):1039-42.

99. Elvinger F, Hansen PJ, Natzke RP. Modulation of function of bovine polymorphonuclear leukocytes and lymphocytes by high temperature in vitro and in vivo. Am J Vet Res. 1991; 52(10):1692-8.

100. Cincović M, Majkić M, Spasojević J, Hristov S, Stanković B, Nakov D, et al. Heat stress of dairy cows in Serbia: Review. Acta Agric Serbica. 2023;28(56):107–25. https://doi.org/10.5937/AASer2356107C

ACCEPTED

101. Zhao S, Min L, Zheng N, Wang J. Effect of Heat Stress on Bacterial Composition and Metabolism in the Rumen of Lactating Dairy Cows. Animals [Internet]. 2019 Nov 5;9(11):925. Available from: https://www.mdpi.com/2076-2615/9/11/925

102. Tajima K, Nonaka I, Higuchi K, Takusari N, Kurihara M, Takenaka A, et al. Influence of high temperature and humidity on rumen bacterial diversity in Holstein heifers. Anaerobe. 2007; 13(2):57-64. doi: 10.1016/j.anaerobe.2006.

103. Plaizier JC, Khafipour E, Li S, Gozho GN, Krause DO. Subacute ruminal acidosis (SARA), endotoxins and health consequences. Anim Feed Sci Technol. 2012;172(1–2):9–21. https://doi.org/10.1016/j.anifeedsci.2011.12.004.

104. Wang X, Li X, Zhao C, Hu P, Chen H, Liu Z, et al. Correlation between composition of the bacterial community and concentration of volatile fatty acids in the rumen during the transition period and ketosis in dairy cows. Appl Environ Microbiol. 2012;78(7):2386–92. doi: 10.1128/AEM.07545-11

105. Klieve A V., Hennessy D, Ouwerkerk D, Forster RJ, Mackie RI, Attwood GT. Establishing populations of Megasphaera elsdenii YE 34 and Butyrivibrio fibrisolvens YE 44 in the rumen of cattle fed high grain diets. J Appl Microbiol. 2003; 95(3):621-30. doi: 10.1046/j.1365-2672.2003.02024.x

106. Schneider P, Sklan D, Chalupa W, Kronfeld DS. Feeding Calcium Salts of Fatty Acids to Lactating Cows. J Dairy Sci. 1988; 8 (71): 2143-2150. https://doi.org/10.3168/jds.S0022-0302(88)79787-8

107. Uyeno Y, Sekiguchi Y, Tajima K, Takenaka A, Kurihara M, Kamagata Y. An rRNA-based analysis for evaluating the effect of heat stress on the rumen microbial composition of Holstein heifers. Anaerobe. 2010; 16(1):27-33. doi: 10.1016/j.anaerobe.2009.04.006

108. Poel M Vander, Collier R, Camacho L, Xiao Y, Compart D, Russo K, et al. Evaluating heat stress response in lactating Holstein cows with supplementation of a feed additive during mid lactation. J Dairy Sci. 2019;102(1):429.

109. Wayman O, Johnson HD, Merilan CP, Berry IL. Effect of Ad Libitum or Force-Feeding of Two Rations on Lactating Dairy Cows Subject to Temperature Stress. J Dairy Sci [Internet]. 1962;45(12):1472–8. Available from: http://dx.doi.org/10.3168/jds.S0022-0302(62)89658-1

110. Fuquay JW, Chapin LT, Brown WH. Short term post-partum heat stress in dairy cows. Int J Biometeorol. 1980;24(2):141–8. https://doi.org/10.1007/BF02253802

111. Beede DK, Collier RJ. Potential Nutritional Strategies for Intensively Managed Cattle during Thermal Stress. J Anim Sci. 1986;62(2):543–54. https://doi.org/10.2527/jas1986.622543x

112. Cowley FC, Barber DG, Houlihan A V., Poppi DP. Immediate and residual effects of heat stress and restricted intake on milk protein and casein composition and energy metabolism. J Dairy Sci.

ACCEPTED

2015;98(4):2356–68. doi: 10.3168/jds.2014-8442

113. Gao ST, Ma L, Zhou Z, Zhou ZK, Baumgard LH, Jiang D, et al. Heat stress negatively affects the transcriptome related to overall metabolism and milk protein synthesis in mammary tissue of midlactating dairy cows. Physiol Genomics. 2019;51(8):400–9. doi: 10.1152/physiolgenomics.00039.2019

114. Novák P, Vokřálová J, Knížková I, Kunc P, Rožnovský J. The influence of high ambient temperatures in particular stages of lactation on milk production of Holstein dairy cows "BIOCLIMATOLOGY AND NATURAL HAZARDS." 2007;(1):978–80.

115. Sakowski T, Kuczyńska B, Puppel K, Metera E, Sloniewski K, Barszczewski J. Relationships between physiological indicators in blood, and their yield, as well as chemical composition of milk obtained from organic dairy cows. J Sci Food Agric. 2012;92(14):2905–12. doi: 10.1002/jsfa.5900

116. Ospina PA, Nydam D V., Stokol T, Overton TR. Associations of elevated nonesterified fatty acids and β-hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the northeastern United States. J Dairy Sci. 2010;93(4):1596–603. doi: 10.3168/jds.2009-2852

117. Kuczyńska B, Puppel K, Gołębiewski M, Wiśniewski K, Przysucha T. Metabolic profile according to the parity and stage of lactation of high-performance Holstein-Friesian cows. Anim Biosci. 2021;34(4):575–83. doi: 10.5713/ajas.20.0018

118. Garcia AB, Angeli N, Machado L, de Cardoso FC, Gonzalez F. Relationships between heat stress and metabolic and milk parameters in dairy cows in southern Brazil. Trop Anim Health Prod. 2015;47(5):889–94. doi: 10.1007/s11250-015-0804-9

119. Kekana TW, Nherera-Chokuda F V., Muya MC, Manyama KM, Lehloenya KC. Milk production and blood metabolites of dairy cattle as influenced by thermal-humidity index. Trop Anim Health Prod. 2018;50(4):921–4. doi: 10.1007/s11250-018-1513-y

120. Roberts T, Chapinal N, LeBlanc SJ, Kelton DF, Dubuc J, Duffield TF. Metabolic parameters in transition cows as indicators for early-lactation culling risk. J Dairy Sci. 2012;95(6):3057–63. https://doi.org/10.3168/jds.2011-4937.

121. Dias MBDC, Leão KM, Do Carmo RM, da Silva MAP, Nicolau ES, Marques TC. Composição do leite e perfil metabólico sanguíneo de vacas holandesas em diferentes ordens de parto e estádios de lactação. Acta Sci - Anim Sci. 2017;39(3):315–21. https://doi.org/10.4025/actascianimsci.v39i3.34807

122. Kim M, Park T, Young Jeong J, Baek Y, Lee HJ. Association between rumen microbiota and marbling score in korean native beef cattle. Animals. 2020;10(4):1–13. doi: 10.3390/ani10040712

ACCEPTED

123. Zhao S, Min L, Zheng N, Wang J. Effect of heat stress on bacterial composition and metabolism in the rumen of lactating dairy cows. Animals. 2019;9(11):925. doi: 10.3390/ani9110925

124. Zhang Q, Zhang S, Cong G, Zhang Y, Madsen MH, Tan B, et al. Effects of soy protein concentrate in starter phase diet on growth performance, blood biochemical indices, carcass traits, immune organ indices and meat quality of broilers. Animals. 2021;11(2):1–13. doi: 10.3390/ani11020281

125. Bardou P, Mariette J, Escudié F, Djemiel C, Klopp C. SOFTWARE Open Access jvenn: an interactive Venn diagram viewer. BMC Bioinformatics. 2014;15(293):1–7. https://doi.org/10.1186/1471-2105-15-293

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Tables and Figures

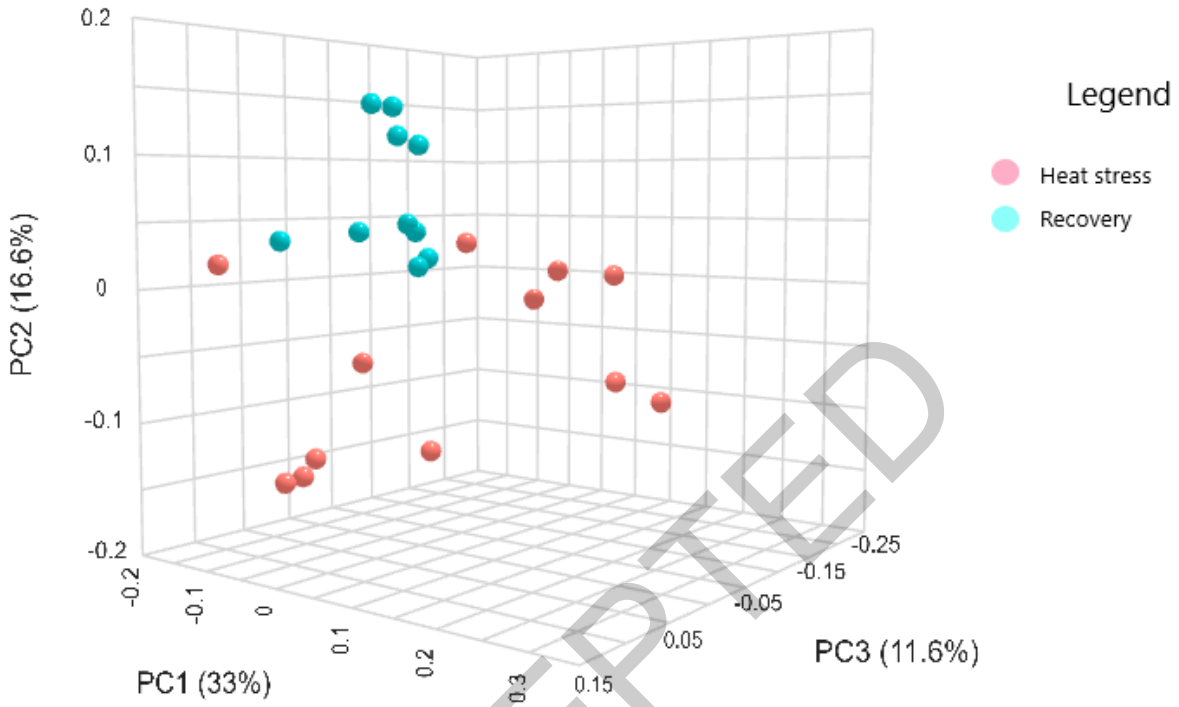

Figure 1. The beta diversity of the rumen bacterial community during both heat stress and subsequent recovery period visualized using Principal coordinate analysis based on Bray-Curtis index dissimilarity with an ellipse at 95% confidence.

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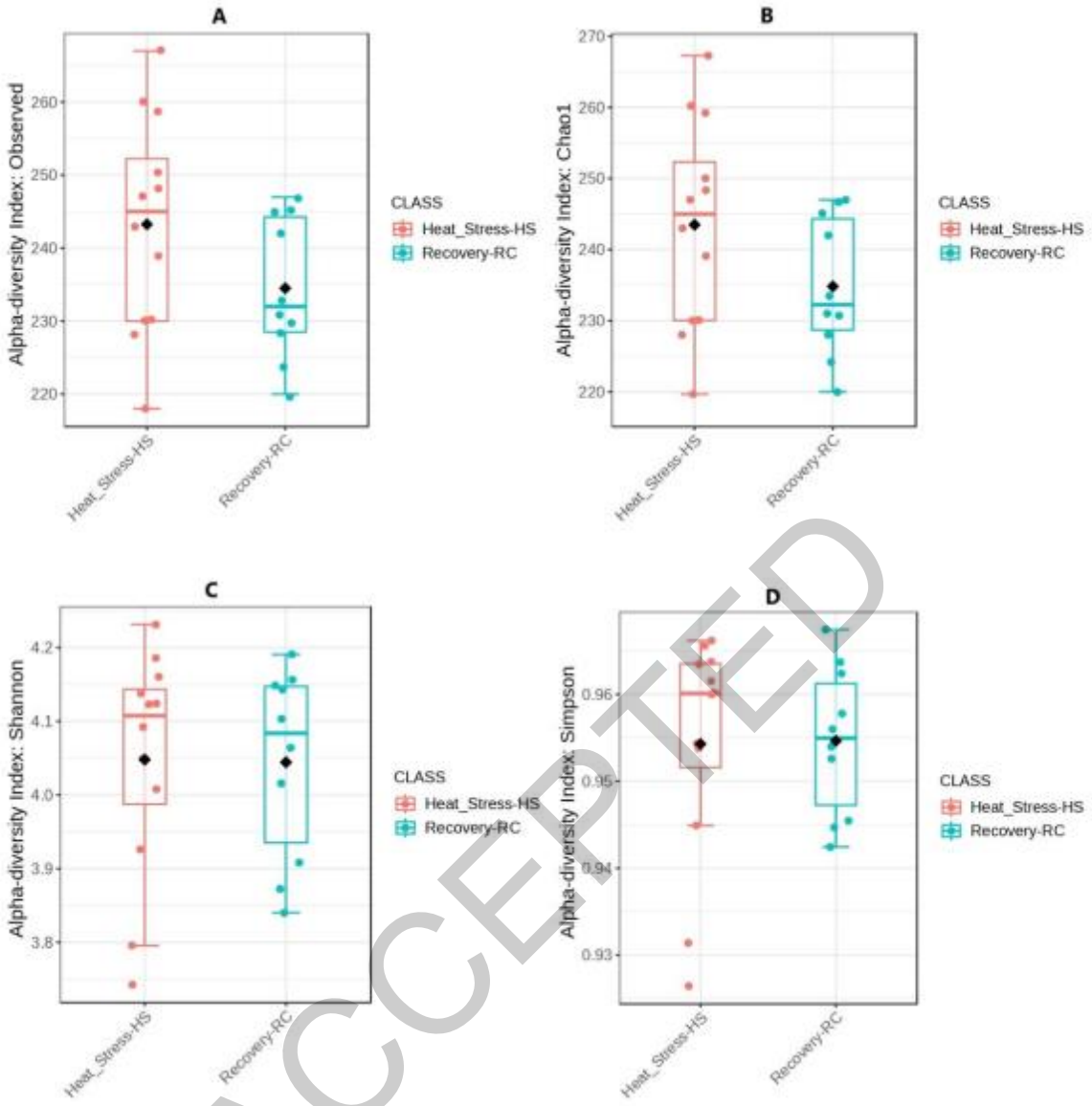

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Figure 2. The boxplot illustrates the alpha diversity indices, including: (A) observed ASVs, (B) Chao1, (C) Shannon, and (D) Simpson indices comparing heat stress and recovery periods. Alpha diversity metrics were visualized using the MicroBiomeAnalyst software. Significant differences were indicated by superscripts a–b ($P < 0.05$).

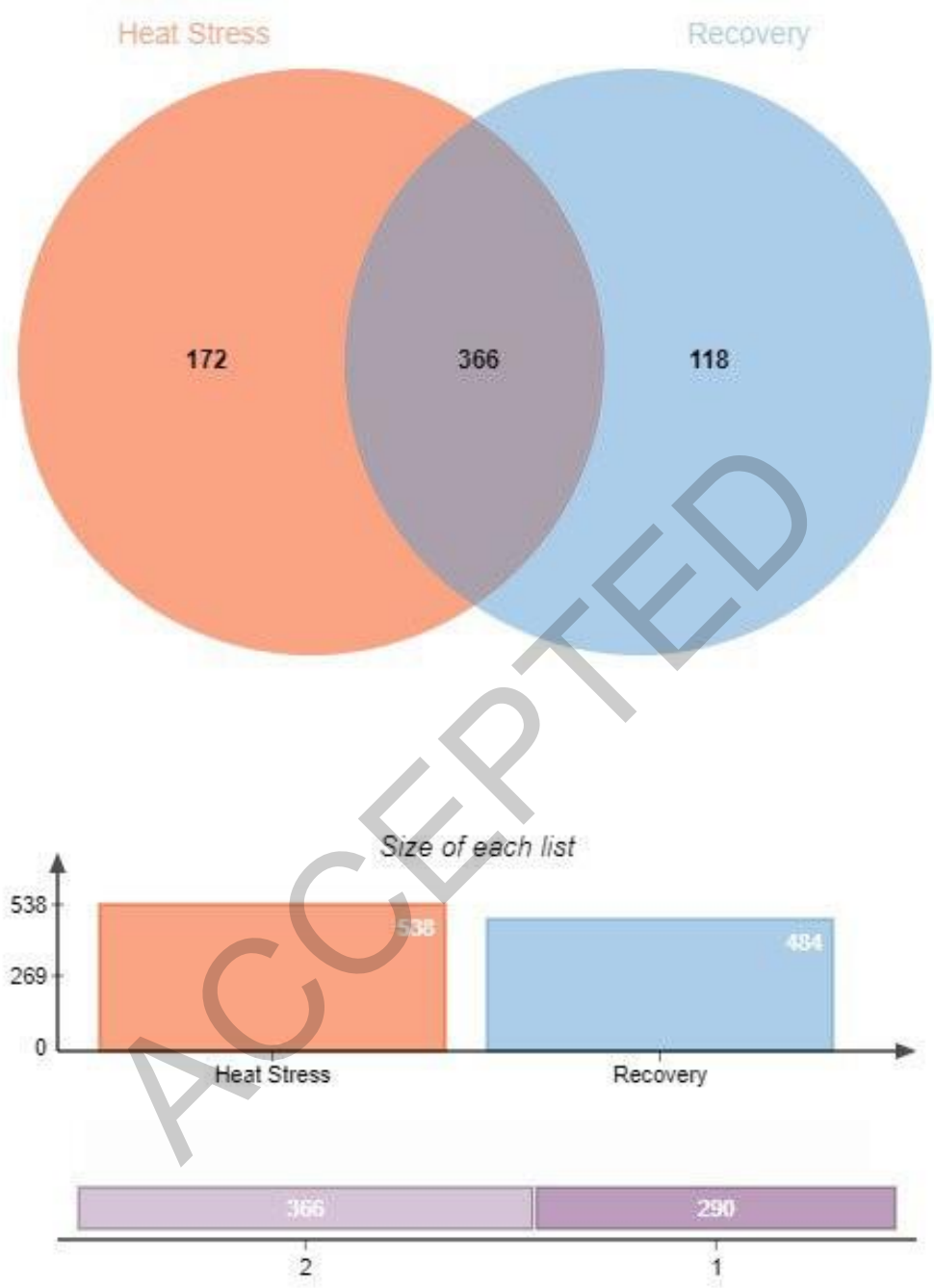

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Figure 3. A Venn diagram represents the unique, shared, and core microbiomes of the rumen during heat stress and recovery periods, alongside a bar graph presenting the size of the representative species of the observed operational taxonomic units per period. A Venn diagram was generated using jVenn software (114).

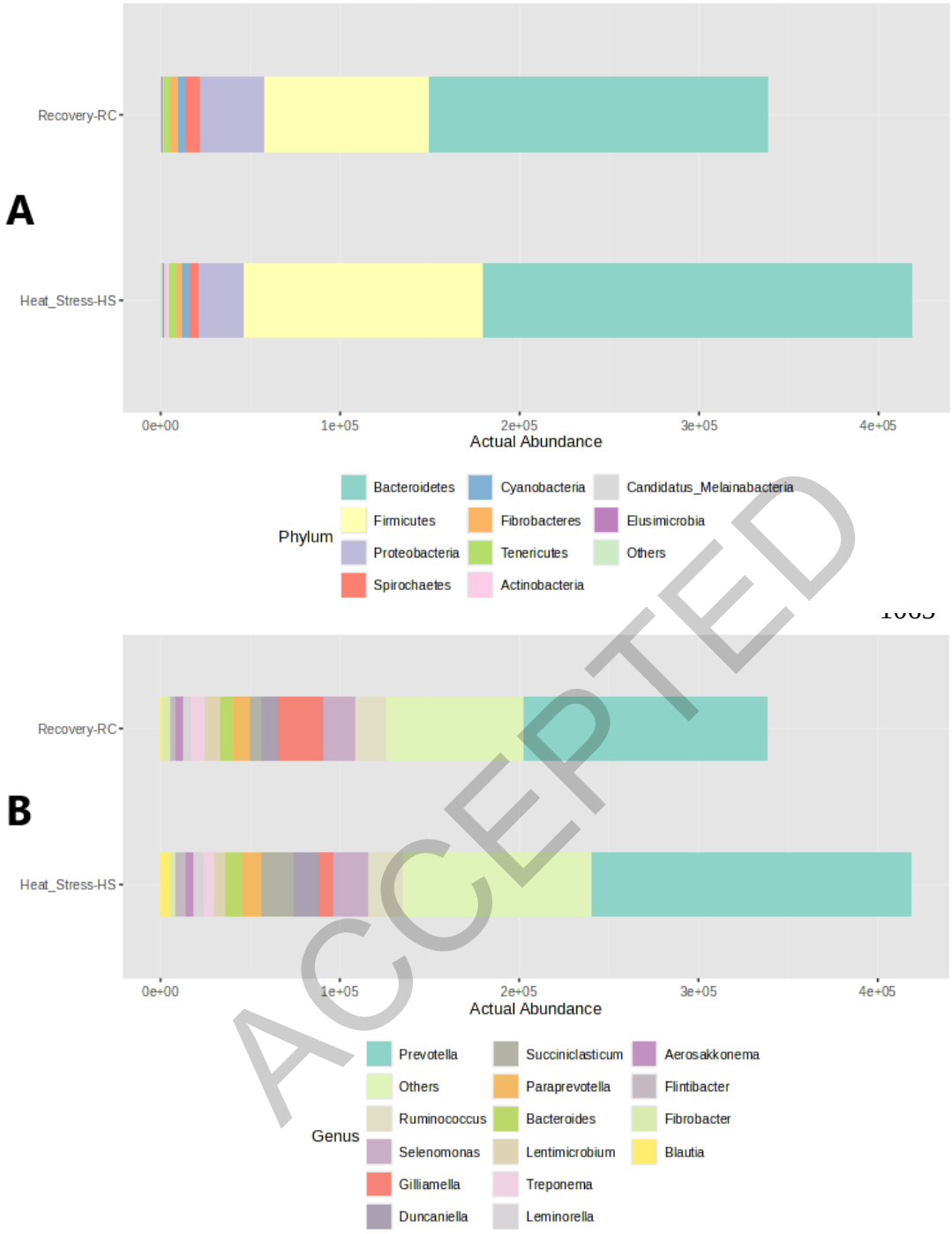

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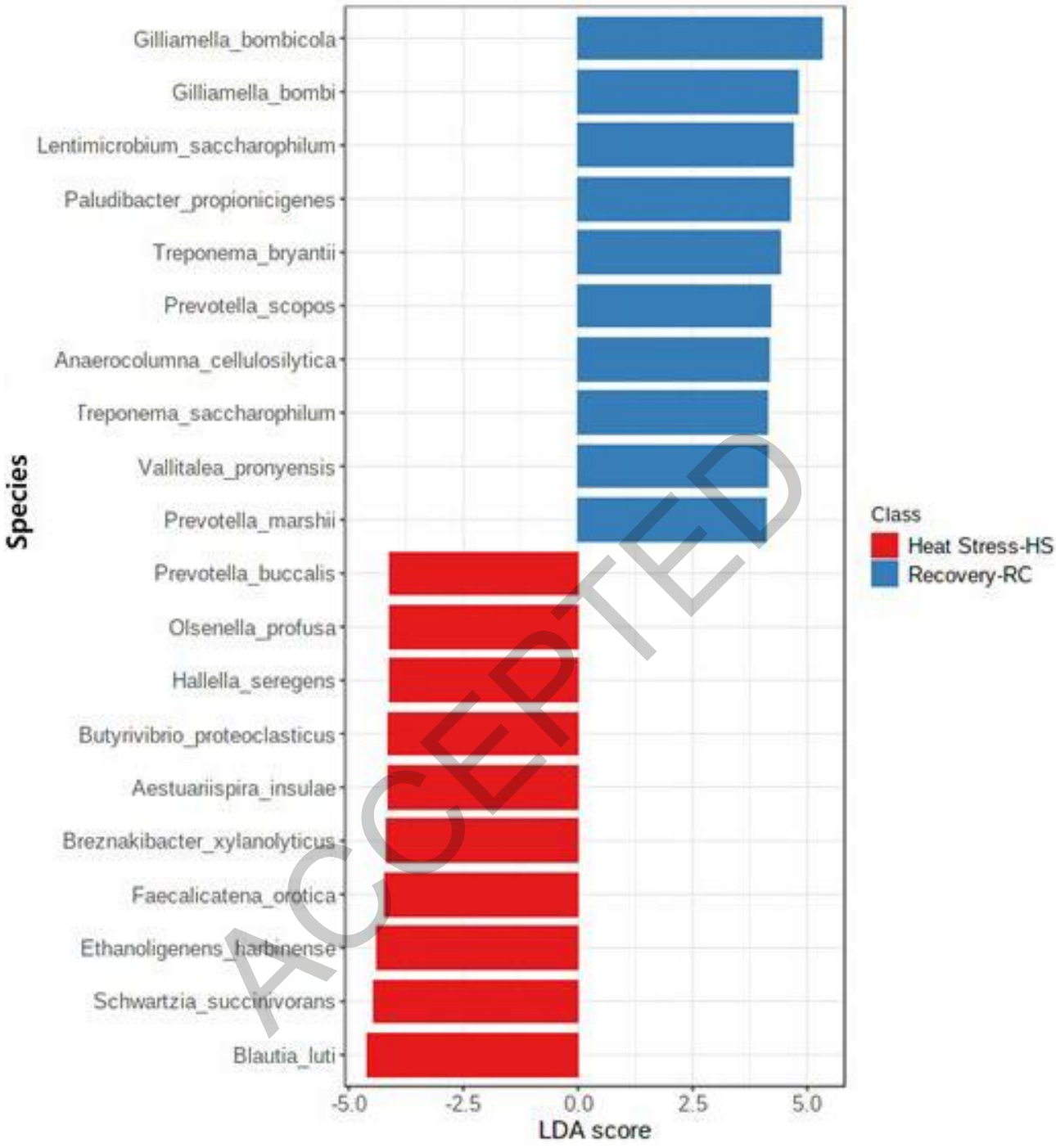
Figure 4. Relative abundances of the observed (A) and (B) genus during heat stress and recovery periods. Asterisks (*) denote a significant difference.



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1091 **Figure 5.** Graphical summary of linear discriminnt analysis effect size during heat stress and recovery periods.

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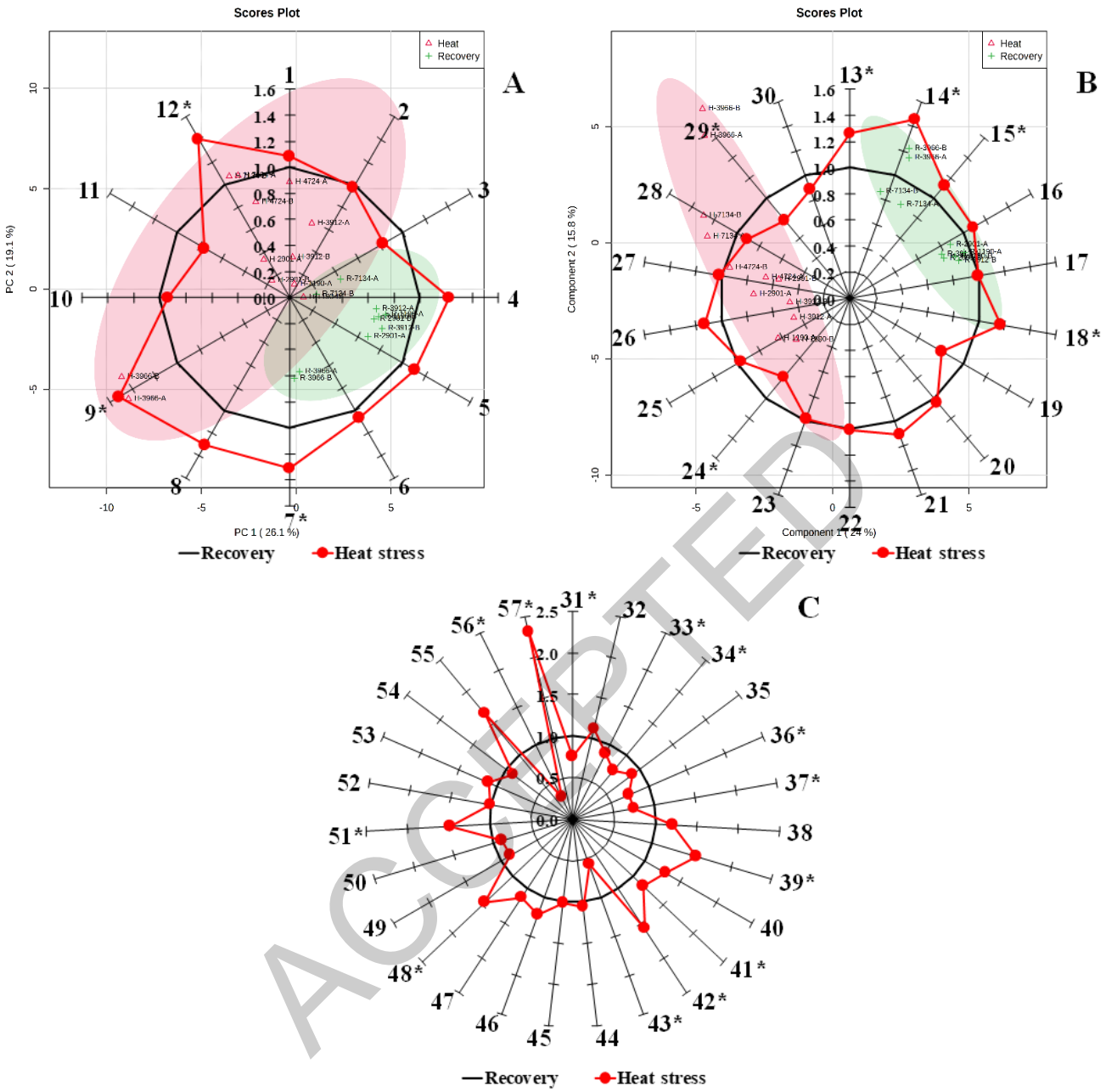

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Figure 6. Star symbol plots represent organic acids (A), fatty acids (B), and amino acids (C) in the sera of the heat stress and recovery groups. The peak numbers correspond to those listed in Table 2. *p < 0.05

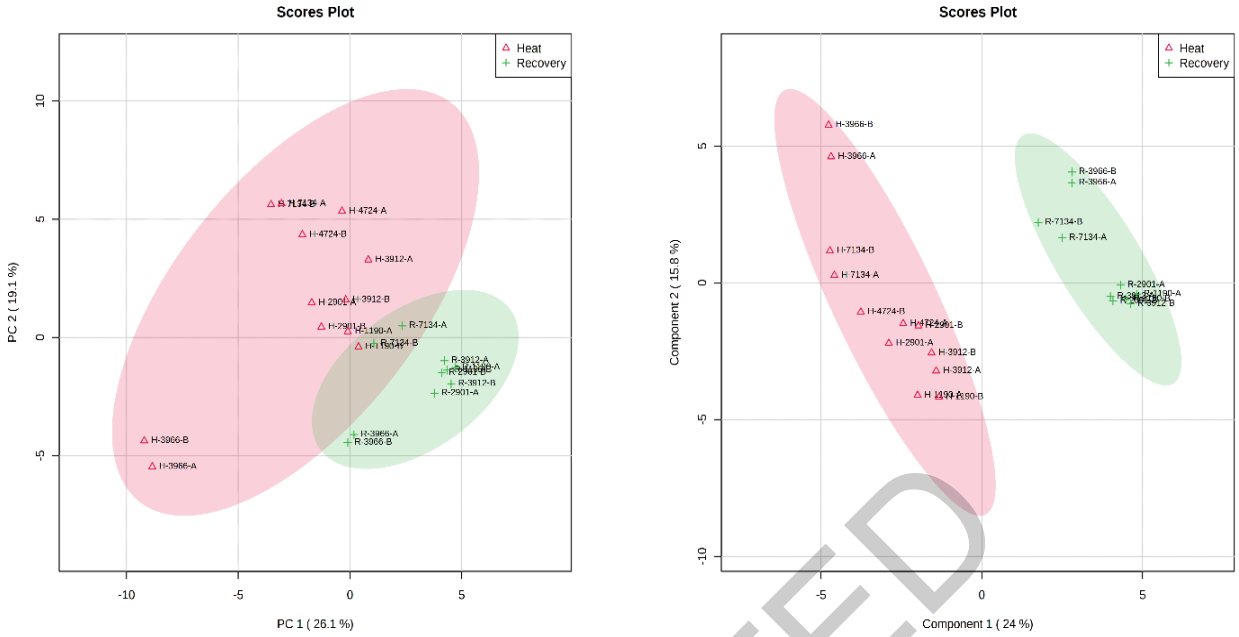

Figure 7. PCA and PLS-DA of heat stress and recovery periods.

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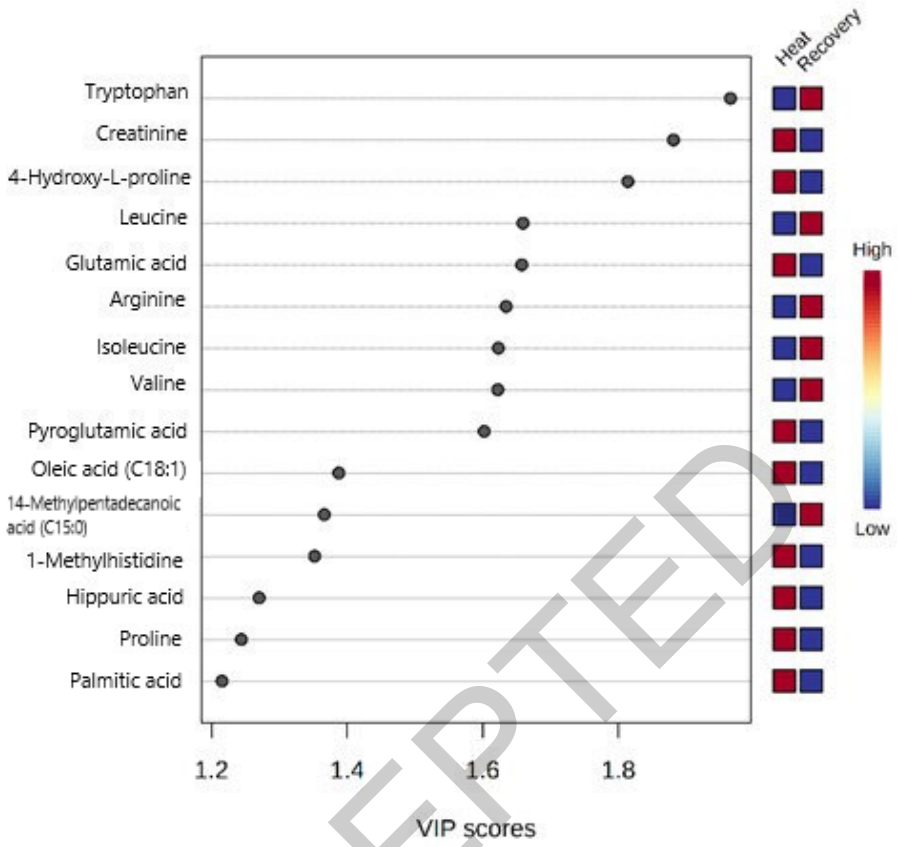

Figure 8. Variable importance analysis of the top 15 metabolites between the heat stress and recovery periods.

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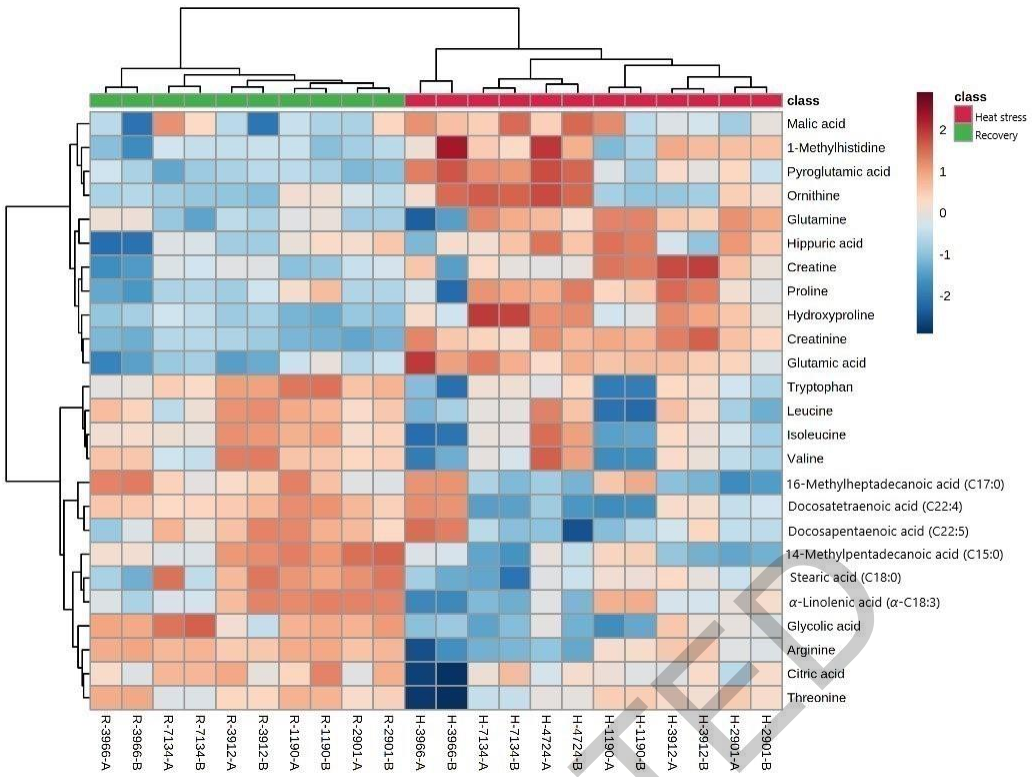

Figure 9. Hierarchical clustering heat maps and group averages of the top 25 metabolites ($p < 0.05$) for the heat stress and the recovery periods.

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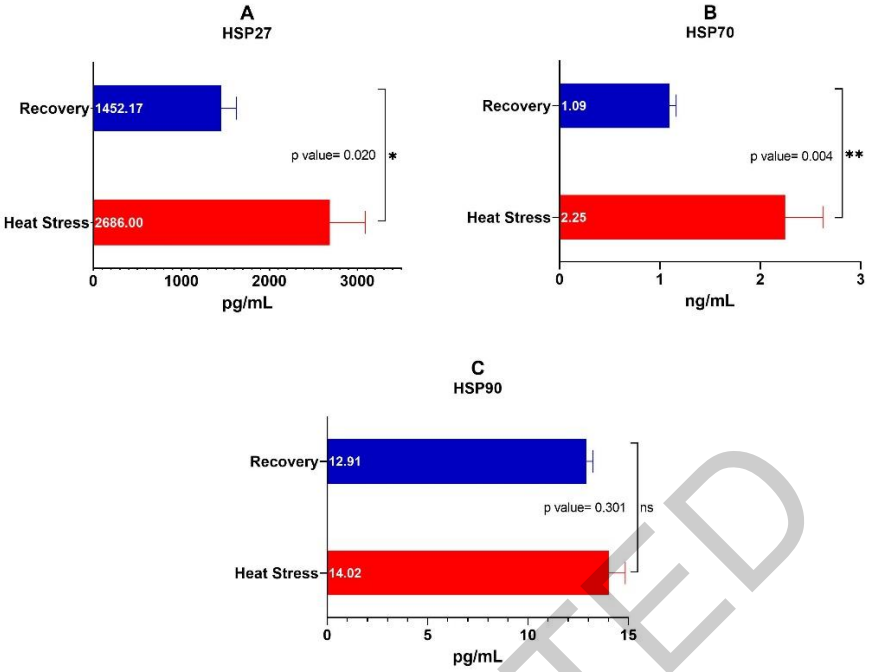

Figure 10. Serum concentrations of (A) HSP27, (B) HSP70, and (C) HSP90 in heat stress (HS) and recovery (RC) periods.

ACCEPTED

Table 1. Ingredients and chemical compositions of total mixed ration fed to the dairy cows.

Ingredients	Composition (% of DM)
Lupine seed	7.50
Whole Cottonseed	10.79
Tall Fescue	29.35
Corn hull	16.10
Corn flake	10.82
Corn silage	10.76
Wheat brain	14.01
Salt	0.33
Vitamin-mineral mix[1]	0.33
Limestone	0.17
Calcium phosphate	0.17
Total	100.00
Chemical compositions (% as DM basis)	
DM (% as fed basis)	44.52
Crude protein	7.24
Crude fiber	24.05
Crude fat	1.70
Ash	5.21
Calcium	0.89
Phosphorus	0.33
NDF	27.50
ADF	15.22

[1]Vitamin-mineral mix contained vit. A 2,650,000 IU, vit. D3 530,000 IU, vit. E 1,050 IU; niacin 10,000 mg; Mn 4,400 mg; Zn 4,400 mg; Fe 13,200 mg; Cu 2,200 mg; iodine 440 mg; and Co 440 mg/kg of Grobic-DC were provided by Bayer Health Care (Leverkusen, Germany).

ACCEPTED

Table 2. Levels of 12 organic acids, 18 fatty acids, 27 amino acids Mann–Whitney test, FDR, and VIP score of PLS-DA

Class	No.	Metabolite	Composition (%, Mean ± SD)		Normalized value[a]	*p*-value	FDR[b]	VIP score
			Heat stress	Recovery				
Organic acid	1	Pyruvic acid	1.3 ± 0.42	1.2 ± 0.31	1.09	0.771	0.897	0.24
	2	Lactic acid	38.3 ± 9.6	39.2 ± 11.9	0.98	0.923	0.974	0.05
	3	Glycolic acid	20.5 ± 1.9	24.6 ± 6.2	0.83	0.050	0.120	0.83
	4	2-Hydroxybutyric acid	0.62 ± 0.27	0.51 ± 0.11	1.22	0.539	0.667	0.44
	5	3-Hydroxypropionic acid	0.75 ± 0.26	0.68 ± 0.16	1.11	0.539	0.667	0.25
	6	3-Hydroxybutyric acid	18.8 ± 8.9	17.6 ± 6.5	1.07	0.821	0.900	0.06
	7	Succinic acid*	0.41 ± 0.10	0.32 ± 0.10	1.31	0.009	0.034	1.06
	8	α-Ketoglutaric acid	0.70 ± 0.45	0.53 ± 0.14	1.30	0.923	0.974	0.35
	9	Malic acid*	0.60 ± 0.20	0.39 ± 0.13	1.52	0.021	0.056	1.19
	10	2-Hydroxyglutaric acid	2.2 ± 0.53	2.4 ± 0.62	0.93	0.346	0.449	0.26
	11	Citric acid	2.1 ± 0.93	2.8 ± 0.67	0.76	0.080	0.164	0.82
	12	Hippuric acid*	13.7 ± 3.4	9.8 ± 1.5	1.40	0.014	0.044	1.27
Fatty acid	13	Myristic acid ($C_{14:0}$) *	0.20 ± 0.029	0.16 ± 0.038	1.26	0.009	0.034	1.21
	14	Palmitoleic acid ($C_{16:1}$) *	0.50 ± 0.18	0.34 ± 0.083	1.46	0.021	0.056	1.16
	15	Palmitic acid ($C_{16:0}$) *	16.8 ± 1.4	15.0 ± 1.6	1.12	0.011	0.040	1.22
	16	γ-Linolenic acid (γ-$C_{18:3}$)	0.55 ± 0.27	0.50 ± 0.13	1.09	0.722	0.858	0.04
	17	Linoleic acid ($C_{18:2}$)	23.6 ± 4.4	23.7 ± 2.1	1.00	0.974	0.974	0.12
	18	Oleic acid ($C_{18:1}$) *	9.8 ± 1.2	8.4 ± 0.57	1.17	0.001	0.007	1.39
	19	α-Linolenic acid (α-$C_{18:3}$)	1.5 ± 0.52	1.9 ± 0.37	0.81	0.123	0.212	0.87
	20	Stearic acid ($C_{18:0}$)	18.2 ± 1.6	17.6 ± 1.0	1.04	0.059	0.130	0.51
	21	Arachidonic acid ($C_{20:4}$)	9.9 ± 1.3	9.0 ± 0.55	1.11	0.093	0.171	0.93
	22	Eicosadienoic acid ($C_{20:2}$)	0.16 ± 0.034	0.16 ± 0.020	1.00	0.974	0.974	0.08
	23	Arachidic acid ($C_{20:0}$)	0.030 ± 0.008	0.031 ± 0.015	0.98	0.254	0.372	0.07
	24	Docosatetraenoic acid ($C_{22:4}$) *	16.9 ± 6.7	21.5 ± 2.8	0.79	0.030	0.078	1.04
	25	Docosapentaenoic acid ($C_{22:5}$)	1.4 ± 0.40	1.5 ± 0.14	0.96	0.203	0.321	0.37
	26	11-Methyldodecanoic acid ($C_{12:0}$)	0.011 ± 0.008	0.010 ± 0.007	1.13	0.821	0.900	0.20
	27	12-Methyltridecanoic acid ($C_{13:0}$)	0.018 ± 0.008	0.018 ± 0.006	1.02	0.974	0.974	0.08
	28	13-Methyltetradecanoic acid ($C_{14:0}$)	0.076 ± 0.026	0.084 ± 0.014	0.91	0.228	0.351	0.56
	29	14-Methylpentadecanoic acid ($C_{15:0}$) *	0.19 ± 0.039	0.24 ± 0.029	0.78	0.003	0.015	1.37
	30	16-Methylheptadecanoic acid ($C_{17:0}$)	0.041 ± 0.013	0.047 ± 0.014	0.89	0.314	0.416	0.49
Amino acid		Alanine	2.5 ± 0.46	2.4 ± 0.51	1.06	0.582	0.706	0.36
		Arginine*	1.2 ± 0.93	4.0 ± 0.66	0.29	< 0.0001	0.000	1.63
		Asparagine	0.64 ± 0.18	0.74 ± 0.069	0.87	0.140	0.235	0.83
		Glutamine	22.4 ± 3.6	20.0 ± 1.6	1.12	0.059	0.130	0.83
		Glutamic acid*	4.0 ± 1.0	2.5 ± 0.38	1.56	< 0.0001	0.000	1.66
		Histidine	2.5 ± 0.53	2.4 ± 0.28	1.02	0.821	0.900	0.02
		Isoleucine*	4.7 ± 0.98	6.4 ± 0.49	0.73	0.002	0.009	1.62
		Leucine*	5.8 ± 0.88	7.6 ± 0.57	0.76	< 0.0001	0.000	1.66
		Lysine	1.7 ± 0.23	1.9 ± 0.34	0.90	0.283	0.384	0.73
		Methionine	1.1 ± 0.15	1.2 ± 0.10	0.90	0.093	0.171	0.90
		Phenylalanine	3.9 ± 1.9	3.5 ± 0.30	1.11	0.069	0.146	0.12
		Proline*	3.2 ± 0.36	2.8 ± 0.26	1.17	0.003	0.013	1.24
		Serine	1.8 ± 0.75	1.7 ± 0.46	1.02	0.283	0.384	0.31
		Threonine*	0.62 ± 0.33	1.1 ± 0.33	0.58	0.017	0.051	1.06
		Tryptophan*	4.1 ± 0.39	5.5 ± 0.25	0.75	< 0.0001	0.000	1.97
		Tyrosine*	1.4 ± 0.16	1.6 ± 0.15	0.88	0.014	0.044	1.17
		Valine*	8.7 ± 1.4	11.5 ± 1.2	0.76	0.0004	0.003	1.62
		1-Methylhistidine*	0.29 ± 0.10	0.19 ± 0.026	1.48	0.002	0.010	1.35
		3-Methylhistidine	0.41 ± 0.10	0.37 ± 0.10	1.11	0.254	0.372	0.49
		α-Aminobutyric acid	1.5 ± 0.57	1.2 ± 0.34	1.29	0.159	0.259	0.72
		Creatine	14.7 ± 2.9	12.0 ± 1.6	1.22	0.050	0.120	1.08
		Creatinine*	2.7 ± 0.39	1.9 ± 0.18	1.46	< 0.0001	0.000	1.88
		Citrulline	3.7 ± 0.46	4.1 ± 0.87	0.90	0.107	0.191	0.52
		Hydroxyproline*	0.50 ± 0.096	0.32 ± 0.044	1.58	< 0.0001	0.000	1.81
		Ornithine	2.9 ± 1.3	1.8 ± 0.30	1.66	0.093	0.171	1.07
		Pipecolic acid	0.038 ± 0.015	0.032 ± 0.006	1.20	0.283	0.384	0.55
	32	Pyroglutamic acid*	3.0 ± 1.4	1.3 ± 0.19	2.31	0.0002	0.002	1.60

*$p < 0.05$

[a] Values normalized to the corresponding control composition mean values.

[b] False discovery rate using the Benjamin–Hochberg method

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Table 3. Effects of heat stress on blood biochemical parameters.

Parameters	Heat stress	Recovery	SEM[1]	*p*-value
Glucose (mg/dL)	58.33[b]	69.30[a]	1.931	0.001
Ketone (mmol/L)	0.79[a]	0.55[b]	0.052	0.005
Blood Urea Nitrogen (mg/dL)	8.25[b]	12.90[a]	0.574	<0.001
Phosphorus (mg/dL)	6.41	6.51	0.321	0.826
Calcium (mg/dL)	8.53	8.86	0.123	0.092
Magnesium (mg/dL)	2.33	2.29	0.053	0.658
Total Protein (g/dL)	10.21[a]	8.02[b]	0.315	0.002
Aspartate aminotransferase (U/L)	107.08[a]	86.60[b]	6.673	0.049
Total Bilirubin (mg/dL)	3.99[a]	0.27[b]	0.608	0.010
Cholesterol (mg/dL)	162.83	199.90	12.874	0.053

[1]SEM = standard error of the mean.

[a,b]Means with different superscripts in the same row differ significantly ($p < 0.05$).

ACCEPTED

Table 4. Effect of heat stress on the complete blood count (CBC).

Parameters[1]	Heat stress	Recovery	SEM[2]	*p*-value
RBC (M/µL)	6.06	5.93	0.175	0.620
HCT (%)	0.29	0.28	0.011	0.828
HGB (g/dL)	9.75	9.42	0.350	0.509
MCV (fL)	47.15	47.17	1.139	0.990
MCH (pg)	16.13	15.83	0.325	0.531
MCHC (g/dL)	34.26	33.57	0.241	0.056
RDW (%)	0.24	0.25	0.004	0.185
RETIC (k/µL)	1.03	1.17	0.196	0.624
WBC (k/µL)	12.76	11.66	1.423	0.590
NEU (%)	0.46	0.46	0.036	0.756
LYM (%)	0.35	0.35	0.027	0.928
MONO (%)	0.14	0.14	0.013	0.961
EOS (%)	0.05[a]	0.03[b]	0.006	0.045
BASO (%)	0.002	0.003	0.001	0.432
NEU (k/µL)	6.22	5.63	1.098	0.720
LYM (k/µL)	4.22	3.96	0.431	0.674
MONO (k/µL)	1.70	1.70	0.237	0.990
EOS (k/µL)	0.58[a]	0.34[b]	0.068	0.023
BASO (k/µL)	0.03	0.03	0.007	0.735
PLT (k/µL)	428.00	293.50	47.681	0.073
MPV (fL)	9.63[b]	10.58[a]	0.142	<0.001
PDW (fL)	7.08[b]	7.80[a]	0.158	0.011
PCT (%)	0.004	0.003	0.001	0.199

[1]Parameters: Total number of erythrocytes (RBC); Hematocrit value: erythrocyte ratio of total blood volume (HCT); Hemoglobin concentration (HGB); Mean erythrocyte volume in the total sample (MCV); Mean hemoglobin volume per RBC count (MCH); Mean hemoglobin concentration of erythrocytes (MCHC); The degree of variation in size of the erythrocyte population (RDW); Reticulocyte count (RETIC); Total number of leukocytes (WBC); Neutrophil percent (%NEU); Lymphocyte percent (%LYM); Monocyte percent (%MONO); Eosinophil percent (%EOS); Basophil percent (%BASO); Neutrophil count (NEU); Lymphocyte count (LYM); Monocyte count (MONO); Eosinophil count (EOS), Basophil count (BASO); Total number of platelets (PLT); Mean platelet volume (MPV); Platelet distribution width; the degree of variation in size of the platelet population (PDW); Plateletcrit value (PCT).

[2]SEM = standard error of the mean.

[a,b]Means with different superscripts in the same row differ significantly ($p < 0.05$).

ACCEPTED

Table 5. Rumen fermentation parameters during the heat stress and recovery periods**.**

Parameters	Heat stress	Recovery	SEM[1]	*p*-value
pH	6.32	6.39	0.072	0.529
[2]NH_3-N (mg/dL)	3.64	2.84	0.531	0.300
Acetate (mmol/L)	57.80	53.22	2.606	0.309
Propionate (mmol/L)	27.76[a]	20.32[b]	1.483	0.006
Butyrate (mmol/L)	18.94	16.99	1.030	0.297
A/P[3]	2.18	2.64	0.129	0.053
Total VFA (mmol/L)	104.50[a]	90.52[b]	3.811	0.030

[1]SEM = standard error of the mean.

[2]NH_3-N = Ammonia-N.

[3]A/P ratio = acetate-to-propionate ratio.

[a,b]Means with different superscripts in the same row differ significantly *($p < 0.05$)*.

ACCEPTED

Table 6. Milk yield and composition of Holstein cows during heat stress and recovery periods.

Parameters	Heat stress	Recovery	SEM[1]	*p*-value
Milk yield, L/day	33.22	31.56	2.420	0.643
Milk fat,%	3.97	4.04	0.164	0.757
Milk protein,%	3.17	3.30	0.090	0.340
SNF[2],%	8.72	9.39	0.133	0.002
MUN[3], mg/day	6.30	10.14	0.256	<.0001

[1]SEM = standard error of the mean.

[2]Solid non-fat = ammonia-N.

[3]MUN = Milk urea nitrogen.

[a,b]Means with different superscripts in the same row differ significantly ($p < 0.05$).

ACCEPTED