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Effects of heat stress on rumen microorganisms and metabolism in different sheep breeds

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Abstract

Background China harbors diverse sheep genetic resources shaped by distinct environmental conditions. Increasing global temperatures and extreme heat events have made heat stress (HS) a major challenge to sheep health and productivity. Different breeds may exhibit distinct adaptive responses to HS, yet the mechanisms underlying breed-specific heat adaptation remain poorly understood. In particular, the integrated responses involving physiological regulation, antioxidant defense, rumen microbial remodeling, and systemic metabolic adaptation under HS have received limited attention.

Methods In this study, four healthy one-year-old sheep of each breed were used: Hu sheep (HuS), Lanping black-boned sheep (LP), and Tibetan sheep (TS). Physiological parameters and serum antioxidant indices were determined under non-heat stress (CON; THI = 62.07) and HS (THI = 85.23) conditions. Meanwhile, 16S rDNA gene sequencing and metabolomic analyses were performed to characterize the rumen microbiota, rumen metabolites, and serum metabolites.

Result Under HS, HuS exhibited significantly lower rectal temperature (RT) and respiratory rate (RR) than LP and TS, while TS showed the highest RT and RR, indicating a greater physiological heat load. Antioxidant responses also differed among breeds, with HuS exhibiting greater glutathione peroxidase

activity, LP showing the highest superoxide dismutase activity, and TS showing the lowest malondialdehyde concentration. In addition, HS markedly reshaped the rumen microbial composition with distinct alterations in key genera including *Prevotella*, *Selenomonas*, and *Succiniclasticum*. Heat stress further altered key rumen metabolites, including L-malic acid, Gamma-Glutamylcysteine, acetic acid, and 8-Oxo-dGMP, together with changes in serum metabolites such as Oxindole, isovaleric acid, and 6-hydroxytrtradeca-8,11-dienoylcarnitine. Integrative analysis revealed strong associations of L-malic acid and Gamma-Glutamylcysteine with serum oxidative stress-related metabolites, while 8-Oxo-dGMP was closely associated with RT and RR. These findings indicate that HuS may have relatively greater heat tolerance, whereas LP and TS are more responsive to heat stress, and provide potential physiological, antioxidant, and metabolic markers for evaluating heat stress sensitivity and breed-specific heat adaptation in sheep.

Keywords: Heat stress; Sheep; Rumen microorganisms; Metabolites; Heat tolerance

Introduction

China possesses abundant sheep genetic resources with extensive geographical distribution. China currently has 110 sheep breeds, with local breeds accounting for as much as 52.7% of the national total. Long-term adaptation to diverse ecological environments has resulted in the formation of locally adapted breeds with distinct phenotypic and physiological characteristics, including Hu sheep (HuS) [1] adapted to high-temperature and high-humidity conditions in low-altitude plains, Lanping black-boned sheep (LP) [2] adapted to relatively humid mountainous environments with low-quality forage, and Tibetan sheep (TS) [3] adapted to low-temperature and hypoxic conditions at high elevations. Although these local breeds have evolved under distinct climatic conditions, the accelerating pace of climate change may expose them to thermal environments beyond their historical adaptive range. However, with ongoing global climate warming and the increasing frequency of extreme heat events,

high ambient temperature has emerged as one of the major environmental stressors limiting sheep productivity and health [4]. As homeothermic animals, sheep have a limited capacity for thermoregulation. When ambient temperature and humidity persistently increase beyond the animal's capacity for heat dissipation, heat stress (HS) can occur [5]. HS imposes a systemic physiological and metabolic burden on sheep, resulting in elevated rectal temperature (RT), respiratory rate (RR), and heart rate (HR), as well as disturbances in redox balance, immune function, energy metabolism, and gastrointestinal function. These changes may reduce feed intake and impair rumen function, ultimately compromising growth performance. Under severe conditions, heat stress can further increase disease susceptibility and mortality, posing substantial economic challenges to the sheep industry [6]. Therefore, improving the ability of sheep to adapt to high-temperature environments and mitigating the adverse effects of HS on animal health and productivity have become important research priorities for the livestock industry in the context of climate change.

The rumen microbiome, often considered an important component of the host's "second genome", plays a pivotal role in nutrient conversion, energy supply, and the maintenance of metabolic homeostasis [7]. Given that sheep breeds have evolved under distinct altitudinal and climatic conditions, long-term environmental adaptation and genetic differentiation may contribute to breed-specific rumen microbial characteristics. Sha et al. [8] found that rumen bacterial groups such as *Rikenellaceae_RC9_gut_group* and *Ruminococcaceae_NK4A214_group* underwent significant changes with increasing altitude, and the microbial functions related to lipid metabolism in the rumen had been enhanced in high-altitude TS. In the HS environment, the reducetion in feed intake and the alterations in the rumen microenvironment will reshape the composition and functional activity of the microbial community, thereby affecting the process of volatile fatty acid (VFA) production, nutrient utilization, and host energy metabolism [9]. Previous studies have demonstrated that different specoes

or breeds exhibit distinct rumen microbial responses mechanisms to HS [10]. It has been reported that HuS exhibit superior heat tolerance compared to the Xinggao sheep, accompanied by higher abundances of fiber-degrading bacteria (*Ruminococcus*, *Fibrobacter*, and the *Bacteroidetes RF16 group*), which may facilitate short-chain fatty acid production and contribute to gastrointestinal homeostasis [11]. However, it remains unclear whether sheep breeds living in different altitudes and climatic environments will develop unique heat-adaptation strategies through coordinated regulation of the rumen microbiome and host metabolic networks. Understanding how breed-specific in rumen microorganisms and metabolic responses contribute to heat adaptation may provide important insights into the underlying differential thermal tolerance mechanisms among sheep breeds.

Metabolomics is a comprehensive approach for elucidating metabolic adaptation responses under environmental stress [12]. Rumen metabolomics can reflect the microbial fermentation and nutrient conversion processes in the rumen, whereas serum metabolomics reflects systemic metabolic responses of the host. Recent studies have demonstrated that the serum and rumen metabolic profiles of sheep are sensitive to breed-specific and environmental differences. For example, serum metabolomics has revealed distinct metabolic signatures between HuS and Dorper sheep with divergent heat tolerance, with differential metabolites mainly involved in protein, carbohydrate, lipid, antioxidant, and inflammatory metabolites, suggesting that breed-specific metabolic remodeling contributes to heat tolerance [13]. Similarly, comparative analyses of HuS introduced from humid low-altitude regions to arid high-altitude environments demonstrated substantial remodeling of rumen microbial communities and metabolites, highlighting the contribution of rumen metabolic plasticity to environmental adaptation [14]. In TS, integrated analyses of rumen microbiota, rumen metabolites, and serum metabolites across different altitudes further showed coordinated changes in amino acid, lipid, carbohydrate, energy, and immune metabolism, suggesting that the rumen-host metabolic axis

plays an important role in adaptation to plateau environments [15]. These findings suggest that animals with different genetic backgrounds may employ breed-specific metabolic strategies to cope with HS. However, whether sheep breeds originating from different altitudinal and climatic environments exhibit distinct rumen and systemic metabolic remodeling patterns under HS, and how these metabolic alterations interact with rumen microbial responses to shape differential heat tolerance, remain poorly understood.

Different sheep breeds have evolved distinct physiological and metabolic characteristics through long-term adaptation to diverse environmental conditions, which may contribute to breed-specific responses to HS. However, studies comparing HS responses among sheep breeds originating from different altitudes under the same high-temperature conditions remain limited. Although previous studies have investigated the physiological responses of sheep to HS, the integrated mechanisms linking breed-specific heat tolerance with antioxidant status, rumen microbial remodeling, and systemic metabolic adaptation remain poorly understood. Therefore, this present study,selected three representative varieties that originated from distinct altitudinal and climatic environments: Hus, Lp and TS. Their physiological parameters, antioxidant status, rumen microbiota, rumen metabolites, and serum metabolites were systematically compared under both heat stess and non-heat stress conditions, and the correlations among different omics layers were further investigated. This study aimed to elucidate breed-specific heat adaptation strategies and their regulatory mechanisms in sheep with distinct genetic backgrounds, thereby providing a theoretical basis for the conservation and utilization of sheep genetic resources, the selective breeding of heat-tolerant sheep breeds, the formulation of HS mitigation strategies, and the establishment of a sustainable sheep production systems under thermal stress in southern China.

Materials and methods

Animals and experimental design

This study was conducted at the Jianghuai Watershed Experimental Station of Anhui Agricultural University (32.57°N, 117.50°E, altitude 70.3 m) from August to October 2022. All of experimental animals in this study were provided by Anhui Huangzhu Animal Husbandry Technology Co., Ltd. The temperature and relative humidity of the sheep barn from August to October were recorded via automatic temperature and humidity loggers (Dalian Cloud Power Technology Co., Ltd., Dalian, China). Three loggers were installed at the front, center, and rear of the barn, each mounted at a height of 1.2 m, and data were recorded at 5-min intervals. In accordance with a previous report, the daily average temperature-humidity index (THI) was used to represent the environmental conditions for each day [16]. During the experimental period, when the THI exceeded 72 for seven consecutive days in August, the sheep were considered to be under HS conditions. Conversely, when the THI remained below 72 for seven consecutive days in October, the sheep were classified as being under non-heat stress (CON) conditions. In this study, healthy one-year-old sheep from HuS, LP, and TS were used (n = 4 per breed). Animals received feed twice a day (9:00am and 5:00pm) and drinking water ad libitum, and each treatment period spanned 10 days under the same management.

Sample collection and preservation

When the sheep were under CON and HS conditions for seven consecutive days, physiological parameters, including RT, RR, and HR, were measured at 14:00 on days 7, 8 and 9, with measurements taken once daily over three consecutive days. Blood samples were collected at 14:00 on day 9 during HS and CON periods. RT was monitored by inserting a digital thermometer (Shang Nong Agriculture Technology Co., Ltd., Wuxi, China) into the rectum (11 cm). The RR was measured by counting thoracoabdominal movements over a 1-min period, with one complete rise and fall defined as a single respiratory cycle. The HR was determined by auscultating the number of heart beats per minute via a

stethoscope placed in the left thoracic region, caudal to the olecranon. On day 9 of the HS and CON periods, 10 mL of blood was collected from the jugular vein of each sheep using non-anticoagulated Vacutainer tubes. Serum was obtained after centrifugation (4°C, 3000×g, 10 min) and subsequently stored at -80°C for biochemical and metabolomic analyses. Simultaneously, rumen fluid samples were collected from each sheep using a stomach tube. Before collecting samples, the initial rumen fluid was discarded, and the collected rumen fluid was filtered through three layers of sterile gauze and transferred to a 50-mL sterile centrifuge tube. The samples were rapidly frozen in liquid nitrogen and then transferred and stored in a -80°C freezer for DNA extraction and metabolomics analysis.

Determination of serum antioxidant indices

Commercially available assay kits (Jianglai Biotechnology Co., Ltd., Shanghai, China) were used to determine serum antioxidant parameters. Serum antioxidant parameters included total antioxidant capacity (T-AOC), malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px).

Ruminal microbial DNA extraction and 16S rRNA amplicon sequencing

Microbial DNA was extracted from rumen fluid samples using the TGuideS96 genomic DNA extraction kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China). The V3-V4 region of the bacterial 16S rRNA gene was amplified using primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The amplicons were purified and quantified, then subjected to paired-end sequencing on the Illumina NovaSeq 6000 platform (Illumina Inc., USA). Paired reads were merged with FLASH (v1.2.11), quality-filtered, and chimera-checked with UCHIME (v8.1). Operational taxonomic units (OTUs) were clustered at 97% sequence similarity using USEARCH (v10.0) and assigned taxonomy against the SILVA database. Alpha diversity was evaluated with the ACE, Shannon, and Simpson indices. Beta diversity was evaluated based on

community distance matrices and visualized by PLS-DA. LEfSe was used to identify bacterial taxa associated with different sheep breeds, with an LDA of >3.0.

Metabolomics sample preparation and data analysis

For each serum and rumen sample, 50 μL was placed in an Eppendorf tube and mixed with 200 μL of a 1:1 methanol/acetonitrile mixture, vortexed for 30 s, and sonicated for 10 min in an ice bath. The mixture was left at -20°C for 1 h and centrifuged at 4°C and 10,000×g for 15 min; the supernatant was pipetted into a clean glass vial. A pooled QC sample was prepared by combining equal volumes from each extract. LC-MS/MS analysis was performed on a Thermo Fisher UHPLC system (CA, USA) equipped with a 2.1 × 100 mm, 1.7 μm UPLC BEH amide column and coupled to a Q Exactive HFX Orbitrap mass spectrometer. Elution used 25 mmol/L ammonium acetate and 25 mmol/L ammonium hydroxide as mobile phase A and acetonitrile as mobile phase B; the autosampler was maintained at 4°C with a 3 μL injection volume. Progenesis QI was used to perform peak alignment, normalization, and identification, with peak areas uniformly corrected using the total peak area normalization method. Metabolite annotation was performed using the BMK Cloud platform (www.biocloud.net) and public databases such as KEGG.

Data analysis

One-way ANOVA with Duncan's post hoc test was applied to physiological and serum antioxidant data. The effects of sheep breed and HS on physiological parameters were evaluated using two-way ANOVA. Microbial community differences were analyzed using Metastats and PERMANOVA where appropriate. Differential metabolites were screened according to VIP > 1, FC > 2 or < 0.5, and $P < 0.05$, followed by false discovery rate (FDR) correction. Hierarchical clustering and fuzzy K-means clustering were performed in R. Spearman correlation analysis was used to evaluate associations among physiological parameters, differential microorganisms, rumen metabolites, serum metabolites,

and antioxidant indicators. The mixOmics R package integrated the multi-omics layers to reveal connections among taxa, metabolites, and phenotypes. All statistics used SPSS 27.0 (IBM Corp., USA), and figures were drawn with GraphPad Prism 8.0.2.

Results

Effects of HS on Environmental Conditions, Physiological Responses, and Serum Antioxidant Capacity in Different Sheep Breeds

Climate conditions that prevailed during the experimental phases of August and October are shown in Fig. 1A-B. Daily averages for RT, RH, and THI in August were 32.14 ± 0.30 ℃, 73.74 ± 1.05, and 85.23 ± 0.35, respectively, while those in October were 17.18 ± 0.32 ℃, 69.64 ±1.86, and 62.07 ± 0.47. Highly significant differences between August and October in terms of temperature and THI were found ($P < 0.05$). In the case of physiological variables, the LP, HuS, and TS showed differential stress responses in the HS period. The physiological parameters, including HR, RR, and RT, showed higher values under HS than under CON ($P < 0.05$). The LP exhibited significantly higher values than HuS in HR, RR, and RT ($P < 0.05$). The TS showed significantly elevated RR and RT compared to HuS ($P < 0.05$). Additionally, the LP had significantly higher HR and significantly lower RR than the TS ($P < 0.05$) (Fig. 1C-E).

Changes in serum antioxidant markers in response to HS are shown in Fig. 1F-J. During HS, CAT activity in TS was the highest among all breeds, while MDA levels and GSH-Px activity in HuS were significantly higher than those in TS, and SOD activity in LP was significantly higher than that in HuS and TS ($P < 0.05$). Furthermore, HS significantly decreased CAT activity, T-AOC, and MDA levels in HuS, while GSH-Px and SOD activities increased significantly. In LP, CAT and SOD activities increased significantly, whereas T-AOC decreased significantly. In TS, CAT and SOD activities increased significantly, whereas T-AOC and MDA levels decreased significantly ($P < 0.05$).

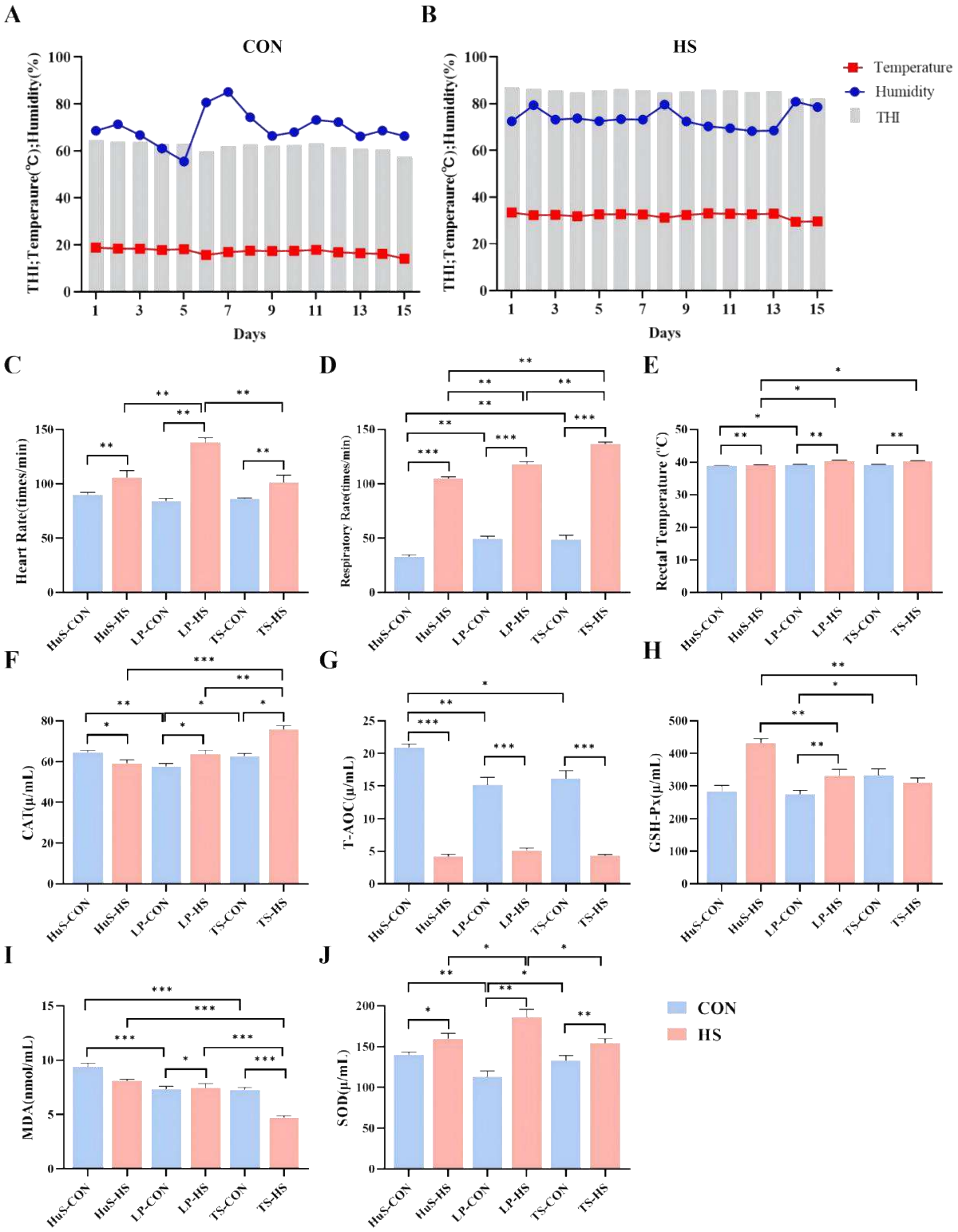


Fig. 1 Effects of HS on Environmental Conditions, Physiological Responses, and Serum Antioxidant Capacity in Different Sheep Breeds. (A-B) Temperature, humidity, and THI values of the sheep barn during CON period of October and HS period of August. (C) Heart rate. (D) Respiratory rate. (E) Rectal temperature. (F) Catalase. (G) Total antioxidant capacity. (H) Glutathione peroxidase. (I)

Malondialdehyde. (J) Superoxide dismutase. Statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Effects of HS on the rumen microbial composition of different sheep breeds

The results of the rumen content microbiota analysis showed that none of the ACE, Chao1 index or Shannon index was affected by HS ($P > 0.05$) (S1). However, the compositions of ruminal phyla and genera were significantly altered (Fig. 2A-B).

At the phylum level, the dominant bacterial phyla for all three sheep breeds were Bacteroidota and Firmicutes (Fig. 2A). To further identify differences in rumen bacteria among the breeds, a Metastats analysis was conducted. The results indicate that, compared with LP and TS, HuS significantly increased the relative abundances of the phyla Proteobacteria, Patescibacteria, Verrucomicrobiota, and Cyanobacteria ($P < 0.05$); HuS significantly reduced the relative abundance of Firmicutes compared with LP, and HuS significantly reduced the relative abundance of Synergistatota compared with TS ($P < 0.05$) (Fig. 2C). Furthermore, HS significantly reduced the relative abundance of Proteobacteria in LP; HS significantly reduced the relative abundance of Bdellovibrionota and significantly increased the relative abundance of Elusimicrobiota in HuS; and HS significantly reduced the relative abundances of Verrucomicrobiota, Proteobacteria, and Cyanobacteria in TS (S1).

Under genus-level HS conditions, the dominant bacterial genus in HuS and TS was *Prevotella*, whereas in LP, the dominant genus shifted to *Quinella* (Fig. 2B). Under HS conditions, compared with LP, HuS showed a significant increase in *Prevotella* and *Candidatus_Saccharimonas* ($P < 0.05$); compared with LP, TS showed a significant increase in *Selenomonas* and *unclassified_Selenomonadaceae*, while *unclassified_F082*, *Prevotella*ceae_UCG_003 were significantly reduced ($P < 0.05$); compared with HuS, *uncultured_rumen_bacterium* and *Prevotella*ceae_UCG_003 in TS were significantly reduced ($P < 0.01$) (Fig. 2D). HS significantly

236 increased the abundance of *Prevotellaceae_UCG_003*, *Christensenellaceae_R_7_group*, and
237 *unclassified_Lachnospiraceae* in LP, while significantly reducing *Succiniclasticum*, *Prevotella*, and
238 *Fibrobacter* ($P < 0.05$); HS significantly increased the abundance of *Selenomonas* and *Prevotella* in
239 HuS, while significantly reducing *Butyrivibrio*, *Saccharofermentans*, *Ruminococcus*,
240 *Rikenellaceae_RC9_gut_group*, and *Veillonellaceae_UCG_001* ($P < 0.05$); HS significantly increased
241 the abundance of *Selenomonas*, *Veillonellaceae_UCG_001*, and *Quinella* in TS, while significantly
242 reducing the abundance of *UCG_002*, *unclassified_F082*, *uncultured_rumen_bacterium*,
243 *Succiniclasticum*, and *unclassified_Clostridia* ($P < 0.05$) (S1). The LDA Score of a total of 23 genus-
244 level rumen microbes was greater than 3 under HS conditions, among which *Succiniclasticum,*
245 *Prevotellaceae_UCG_003*, *Stenotrophomonas*, and *Elusimicrobium* were significantly enriched in
246 HuS; *Fretibacterium*, *unclassified_F082*, *Bacteroidales_bacterium_Bact_22*, and *probable_genus_10*
247 were significantly enriched in LP; *Selenomonas*, *Prevotella_7*, *Lachnoclostridium*, and *Shuttleworthia*
248 were significantly enriched in TS (Fig. 2E).

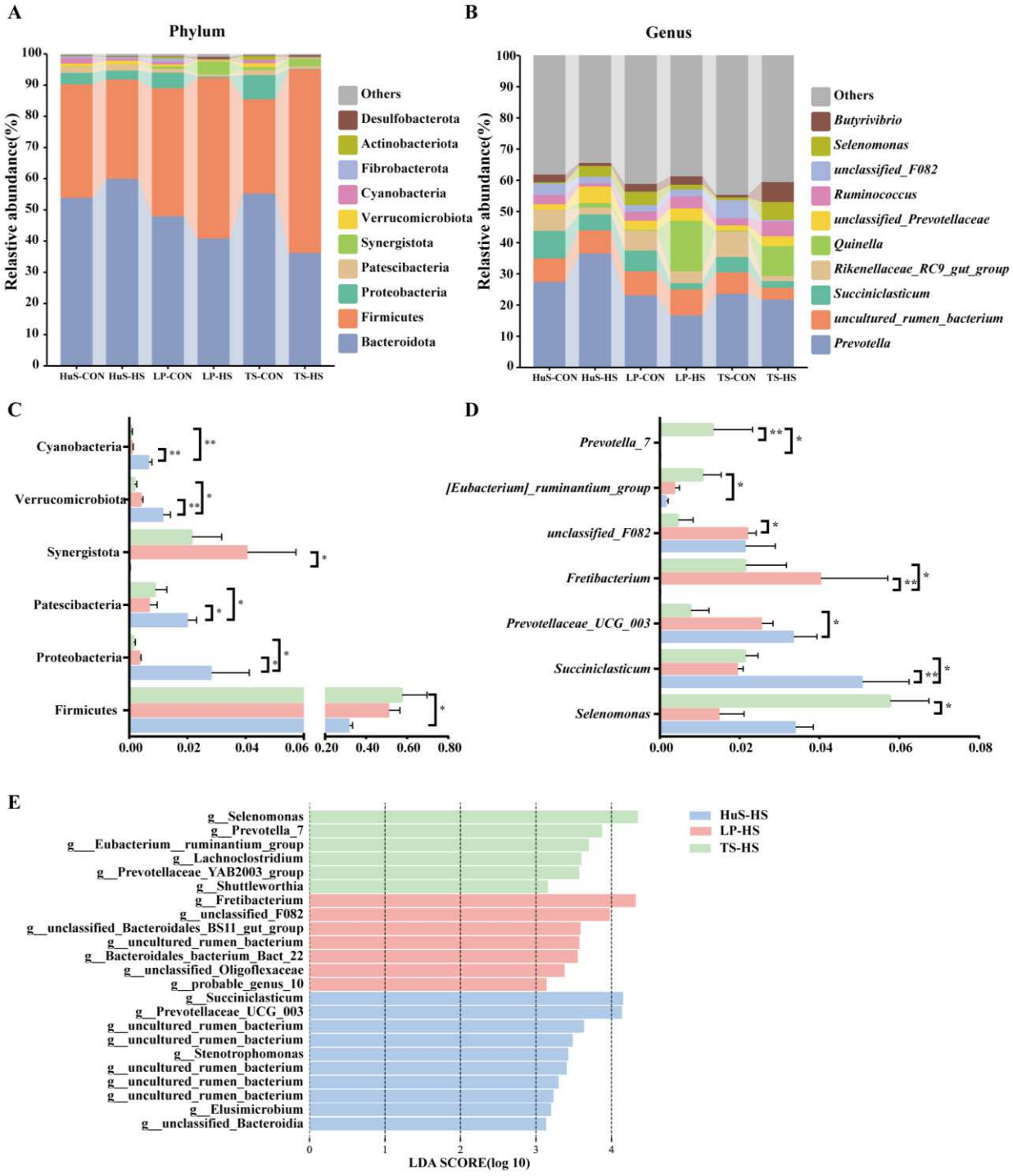


Fig. 2 Effects of HS on rumen microbial community composition and genus-level abundance in different sheep breeds. (A-B) The composition of the microbiota at the phylum and the genus level. (C-D) Differentially abundant phyla and genera of rumen microbiota among sheep breeds under HS. (E) LEfSe analysis at the genus level. Statistical significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Effects of HS on rumen and serum metabolite composition in different sheep breeds

PLS-DA and OPLS-DA analyses showed that during the CON period, neither rumen nor serum

metabolite profiles exhibited clear separation among HuS, LP, and TS, whereas distinct separation among the three breeds was observed under HS, indicating that HS markedly altered both rumen and systemic metabolic profiles (Fig. 3A-D, Fig. 4A-D). In the rumen, volcano plot analysis identified 449, 645, and 94 differentially abundant metabolites between HuS-LP, HuS-TS, and LP-TS, respectively, under HS (Fig. 3E, G, I). After metabolite annotation and screening for HS-related metabolites, 178 differentially expressed metabolites were identified in the HuS-HS vs. LP-HS comparison, including beta-Carotene, Acetic acid, L-malic acid, and Pantothenic Acid, which were significantly enriched in pathways related to vitamin digestion and absorption, methane metabolism, beta-Alanine metabolism, and carbohydrate digestion and absorption ($P < 0.05$) (Fig. 3F). In the HuS-HS vs. TS-HS comparison, 170 differential metabolites, including Uracil, D-Galactose, L-Phenylalanine, and Thymine, were significantly enriched in pyrimidine metabolism, carbohydrate digestion and absorption, methane metabolism, and mineral absorption ($P < 0.05$) (Fig. 3H). In the LP-HS vs. TS-HS comparison, 42 differential metabolites, including Cholesterol, Androstenedione, Tetrahydrocorticosterone, and beta-Carotene, were significantly enriched in cancer-related pathways, steroid degradation, ovarian steroidogenesis, and steroid hormone biosynthesis ($P < 0.05$) (Fig. 3J). Overall, differential rumen metabolites among the three breeds were mainly associated with amino acid, fatty acid, bile acid, and carbohydrate metabolism, with significant enrichment in HS-related pathways such as riboflavin metabolism, arginine biosynthesis, and galactose metabolism (S2). Similarly, serum metabolomic analysis identified 229, 130, and 299 differentially abundant metabolites between HuS-LP, HuS-TS, and LP-TS, respectively, under HS (Fig. 4E, G, I). Following metabolite annotation and screening, 97 differential metabolites were identified in the HuS-HS vs. LP-HS comparison, including 18-Oxooleate, Oleic acid, Urocortisol, and L-Glutamine, which were significantly enriched in beta-Alanine metabolism, cutin, suberine and wax biosynthesis, steroid hormone biosynthesis, and nitrogen

metabolism ($P < 0.05$) (Fig. 4F). In the HuS-HS vs. TS-HS comparison, 58 differential metabolites, including Chitobiose, Thiamine, Isovaleric acid, Inosinic acid, and Pyridoxamine, were significantly enriched in ABC transporters, biosynthesis of alkaloids derived from histidine and purine, and vitamin digestion and absorption ($P < 0.05$) (Fig. 4H). In the LP-HS vs. TS-HS comparison, 174 differential metabolites, including Leukotriene D4, Prostaglandin F2alpha, Uric acid, Glycocholic acid, and 18-Oxooleate, were significantly enriched in arachidonic acid metabolism, bile secretion, serotonergic synapse, and cutin, suberine and wax biosynthesis ($P < 0.05$) (Fig. 4J). Overall, serum differential metabolites were primarily involved in amino acid, steroid, bile acid, and vitamin metabolism, with significant enrichment in phenylalanine metabolism, amino acid biosynthesis, steroid hormone biosynthesis, and primary bile acid biosynthesis (S3). These findings demonstrate that HS induced substantial and breed-specific metabolic reprogramming in both the rumen and circulation, involving nutrient metabolism, microbial fermentation-related pathways, antioxidant-related metabolism, and endocrine and lipid metabolic processes.

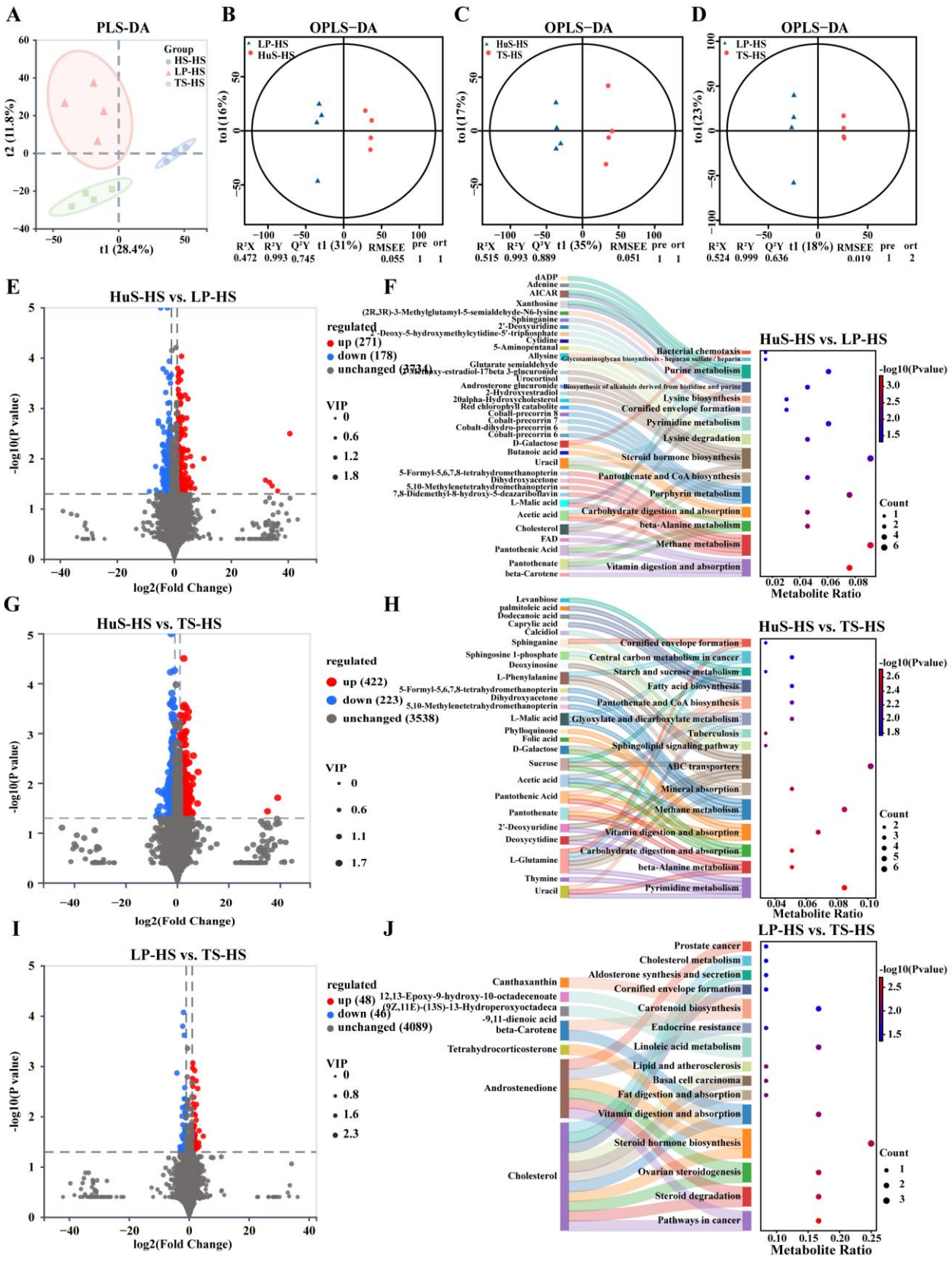


Fig. 3 Effects of HS on rumen metabolite composition in different sheep breeds. (A-D) PLS-DA and OPLS-DA score plots of HuS, LP, and TS in the HS period. (E, G, I) Volcanic plots of HuS, LP, and TS in the HS period. (F, H, J) Sankey dot pathway enrichment of HuS, LP, and TS in the HS period.

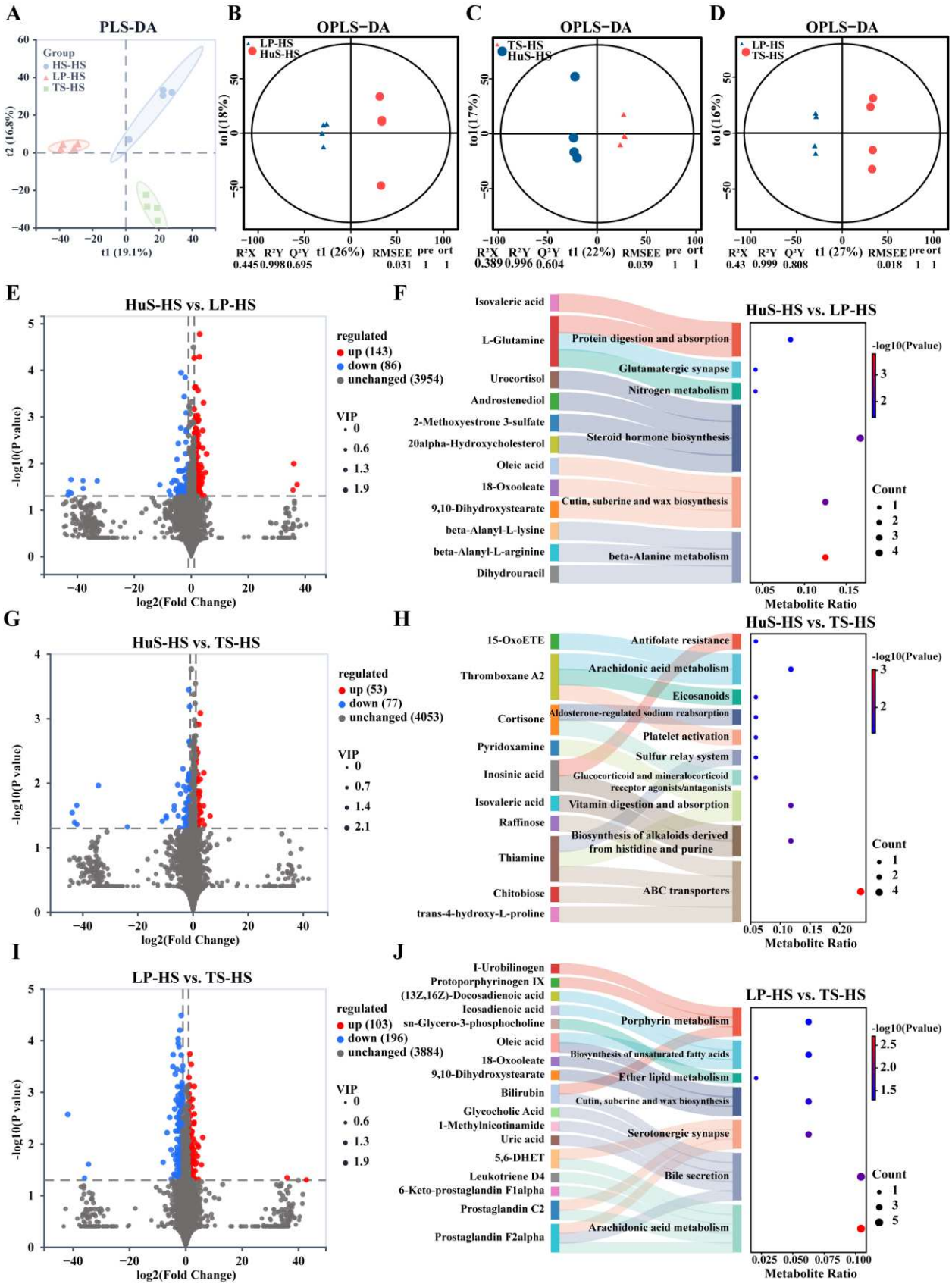


Fig. 4 Effects of HS on serum metabolite composition in different sheep breeds. (A-D) PLS-DA and OPLS-DA score plots of HuS, LP, and TS in the HS period. (E, G, I) Volcanic plots of HuS, LP, and TS in the HS period. (F, H, J) Sankey dot pathway enrichment of HuS, LP, and TS in the HS period.

K-means clustering analysis was performed to characterize the patterns of differential rumen and serum metabolites among the three sheep breeds under HS conditions. Differential rumen metabolites were clearly classified into four clusters (Fig. 5A). Cluster 1 mainly comprised Fatty Acyls, Carboxylic acids and derivatives, and Steroids and steroid derivatives, with metabolites such as Acetic acid, Octadecenoic acid, 8-Oxo-dGMP, Gamma-Glutamylcysteine, and Tetradecenoylcarnitine showing significantly lower abundances in HuS than in LP and TS. Cluster 2 mainly consisted of Fatty Acyls, Carboxylic acids and derivatives, and Glycerophospholipids, and exhibited a gradual increasing trend across the three breeds, with Dihydroxyacetone, Uracil, Bilirubin diglucuronide, and Pantothenate among the representative metabolite (Fig. 6A). Cluster 3 contained metabolites with significantly higher abundances in LP than in HuS and TS, such as (9Z,11E)-(13S)-13-Hydroperoxyoctadeca-9,11-dienoic acid, whereas Cluster 4 showed a decreasing trend and was mainly composed of Fatty Acyls, Prenol lipids, and Glycerophospholipids, including Tetradecanedioic acid, Sphinganine, 3-Hydroxyisobutyric acid, and 12-hydroxyicosanoic acid ($P < 0.05$) (Fig. 5B, 6B). Similarly, differential serum metabolites were also divided into four distinct clusters (Fig. 5C), with the metabolites predominantly belonging to Carboxylic acids and derivatives, Fatty Acyls, and Glycerophospholipids. Cluster 1 metabolites, including 3-Formyl-6-hydroxyindole, Glutaminyllysine, 13,14-dihydro PGF-1α, and Phenylalanylproline, exhibited significantly lower abundances in HuS than in LP and TS, whereas Cluster 2 metabolites, including Isovaleric acid, 6-hydroxytrtradeca-8,11-dienoylcarnitine, 5-Hydroxytryptophol sulfate, and N-Methylserotonin, were significantly more abundant in HuS (Fig. 6A). In Cluster 3, metabolites such as 9,10,13-Trihydroxystearic acid, LysoPE(0:0/20:0), CPA(16:0/0:0), and Leukotriene D4 showed significantly lower abundances in LP than in HuS and TS, while Cluster 4 contained metabolites, including 3-Dehydroshikimate, Chitobiose, and 2-Octaprenyl-6-hydroxyphenol, that were also significantly less abundant in LP ($P < 0.05$) (Fig. 5D). Collectively,

K-means clustering revealed distinct breed-specific metabolic patterns in both the rumen and serum during HS, with differential metabolites predominantly involving fatty acid, carboxylic acid, glycerophospholipid, steroid, and related lipid metabolic processes, further indicating that the three sheep breeds employed distinct metabolic strategies in response to HS.

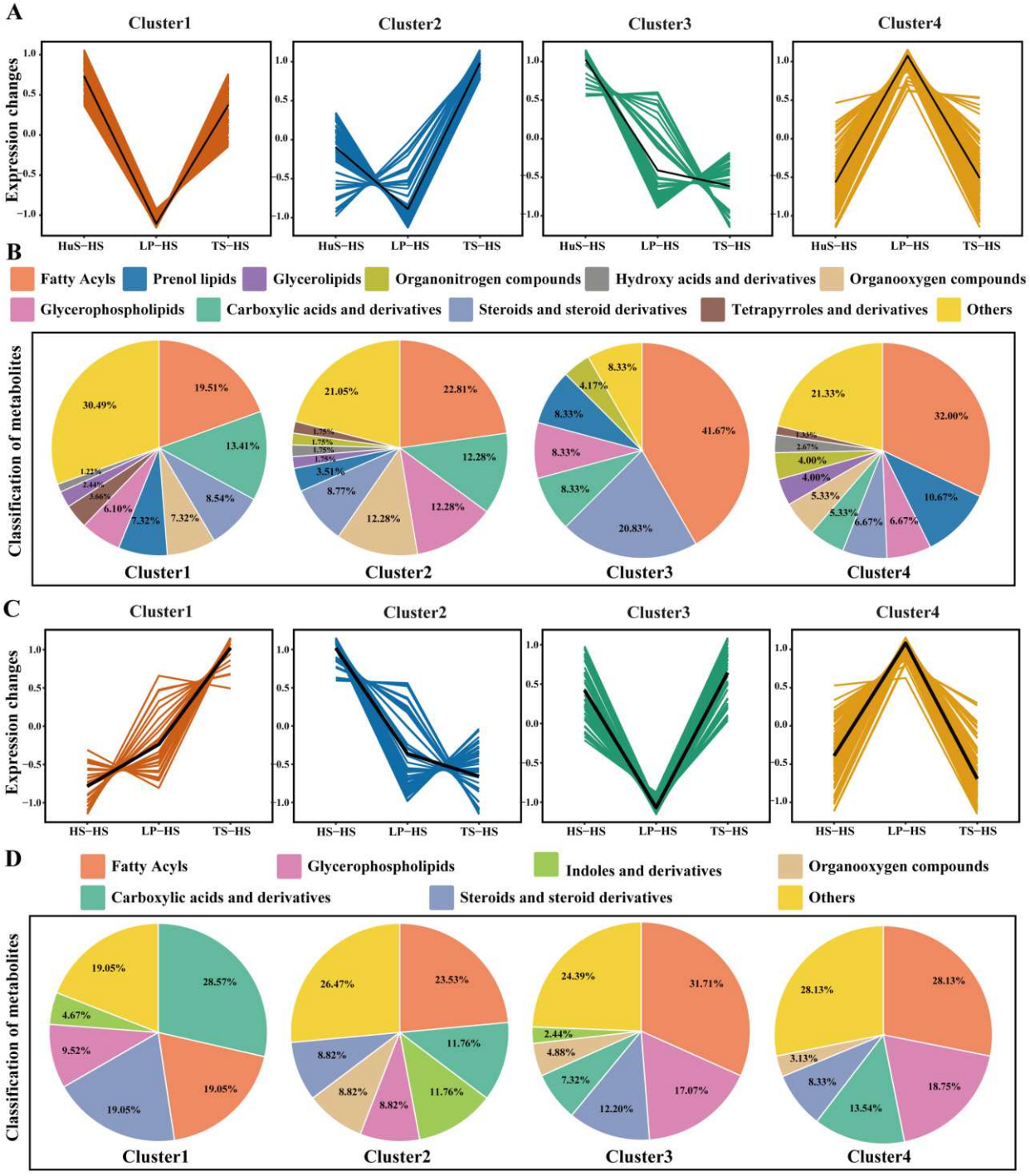


Fig. 5 K-means analysis of rumen and serum metabolites in different sheep breeds under HS. (A, C) K-means clustering analysis of differentially abundant serum and rumen metabolites across different

sheep breeds under HS. (B, D) K-means cluster analysis of serum and rumen metabolite classification in different sheep breeds under HS.

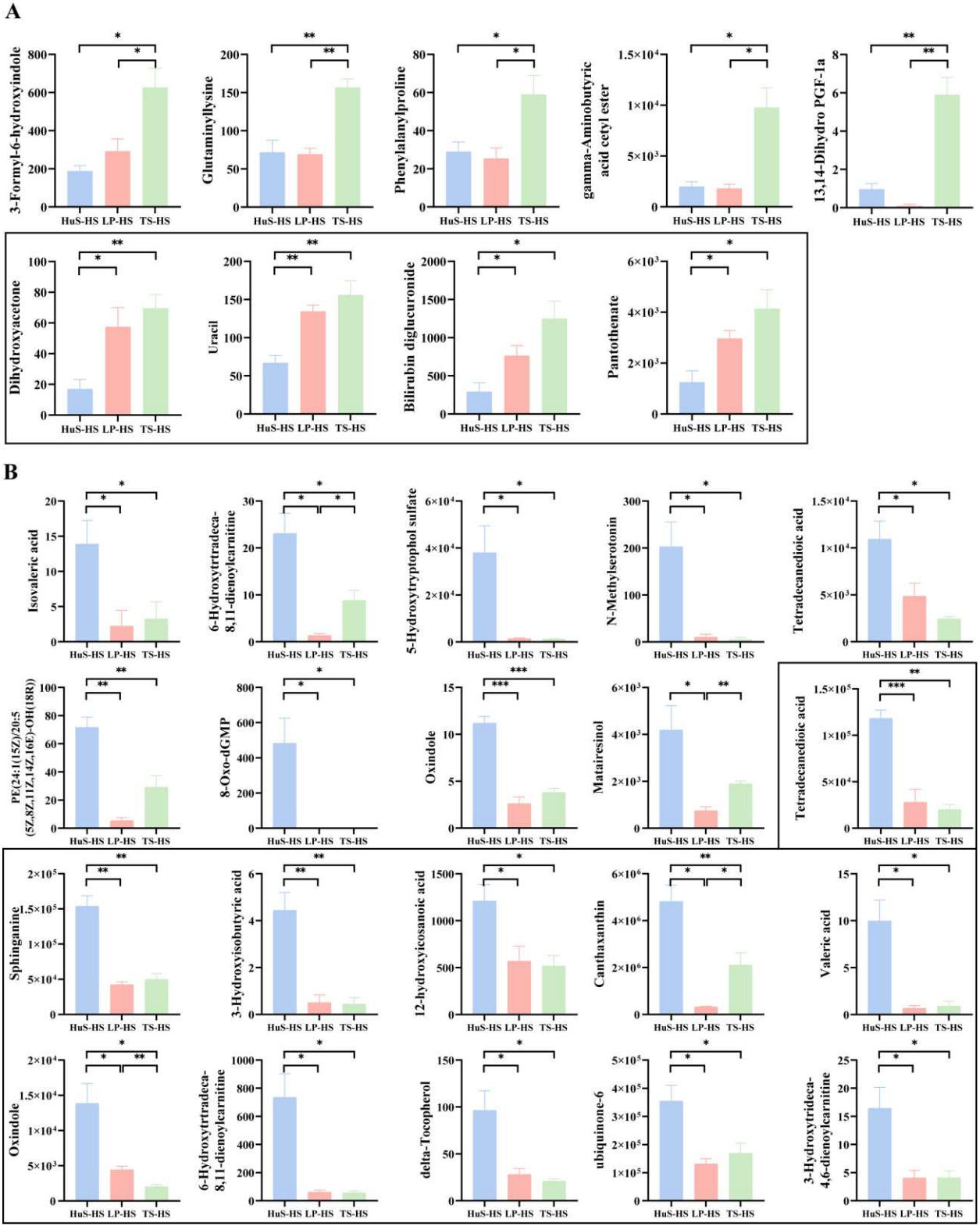


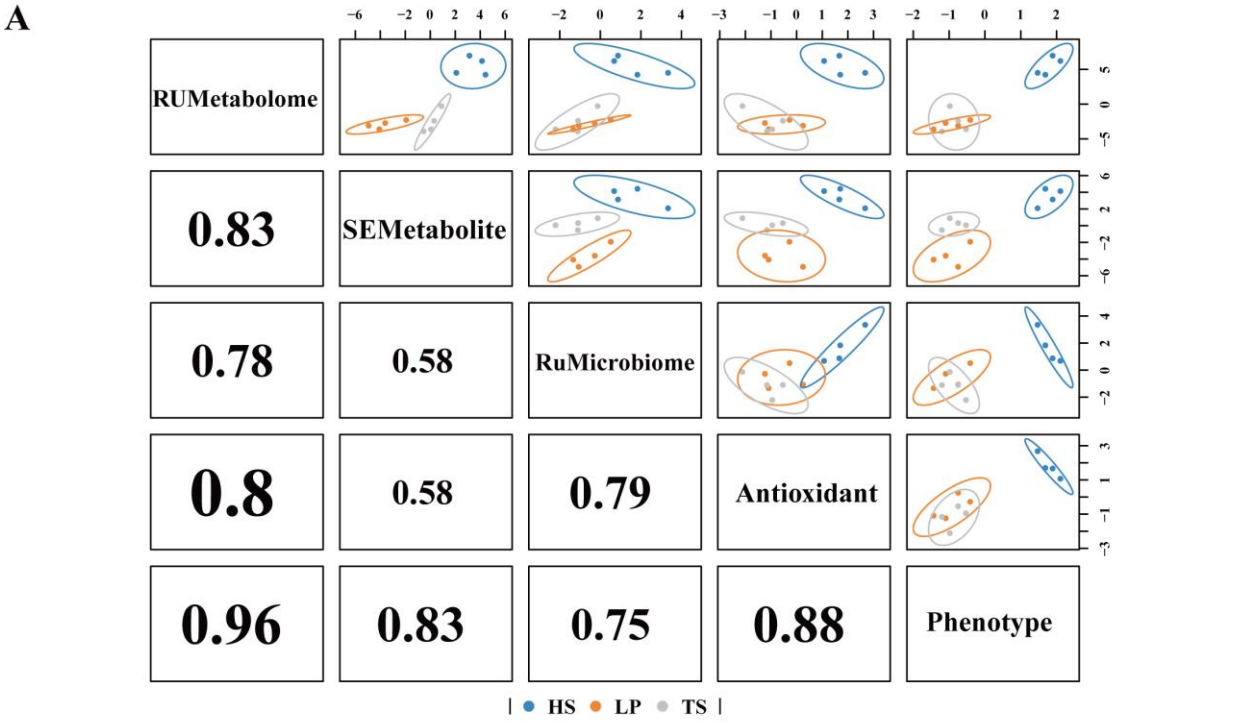
Fig. 6 Differences in rumen and serum metabolite abundance among different sheep breeds under HS. (A) Progressively increasing rumen and serum metabolites in HuS, LP, and TS under HS. (B)

335 Progressively decreasing rumen and serum metabolites in HuS, LP, and TS under HS. Statistical
336 significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The substances enclosed in the black boxes are
337 serum metabolites; the rest are rumen metabolites.

338 **Multi-omics analysis of different sheep breeds under HS**

339 To elucidate the relationships among biological traits across different regulatory levels, an
340 integrative correlation analysis was performed by combining the rumen metabolome, serum
341 metabolome, rumen microbiome, antioxidant parameters and heat-stress-related phenotypic traits.
342 Using a correlation threshold of $|r| > 0.8$, a broad correlation network was constructed among features
343 from different omics layers. The strongest association was detected between the rumen metabolome
344 and physiological phenotypes ($r = 0.96$), followed by antioxidant indices and physiological phenotypes
345 ($r = 0.88$), serum metabolome and physiological phenotypes ($r = 0.83$), and rumen metabolome and
346 serum metabolome ($r = 0.83$) (Fig. 7A). Among the trait–metabolite pairs, RR was negatively
347 correlated with Oxindole, RT was positively correlated with dihydroxyacetone, and HR was negatively
348 correlated with leukotriene D4. Antioxidant indices were also significantly associated with multiple
349 metabolites and rumen microbes; for example, GSH-Px was positively correlated with 3-
350 hydroxyisobutyric acid, CAT was negatively correlated with pregnanetriolone, and MDA was
351 positively correlated with Oxindole. Extensive and significant correlations were identified between
352 microbial taxa and rumen/serum metabolites, indicating that alterations in the rumen microbiota may
353 drive metabolic remodeling during adaptation to heat stress. At the genus level, *Fretibacterium* was
354 significantly negatively correlated with Oxindole, sphinganine, canthaxanthin and isovaleric acid,
355 whereas it was positively correlated with Gamma-Glutamylcysteine and L-malic acid. *Prevotella_7*
356 exhibited a significant positive correlation with 13,14-dihydro PGF-1α, while *Succiniclasticum* was
357 negatively correlated with acetic acid and dihydroxyacetone but positively correlated with valeric acid

and tetradecanedioic acid. Collectively, these findings indicate that heat-stress adaptation in sheep is not governed by a single biological process but rather by multi-level regulatory mechanisms involving the coordinated interplay among the rumen microbial community, the rumen metabolic network, the circulating metabolic profile and the antioxidant defense system (Fig. 7B).


A
RUMetabolome
SEMetabolite
RuMicrobiome
Antioxidant
Phenotype
0.83
0.78
0.58
0.8
0.58
0.79
0.96
0.83
0.75
0.88
HS
LP
TS

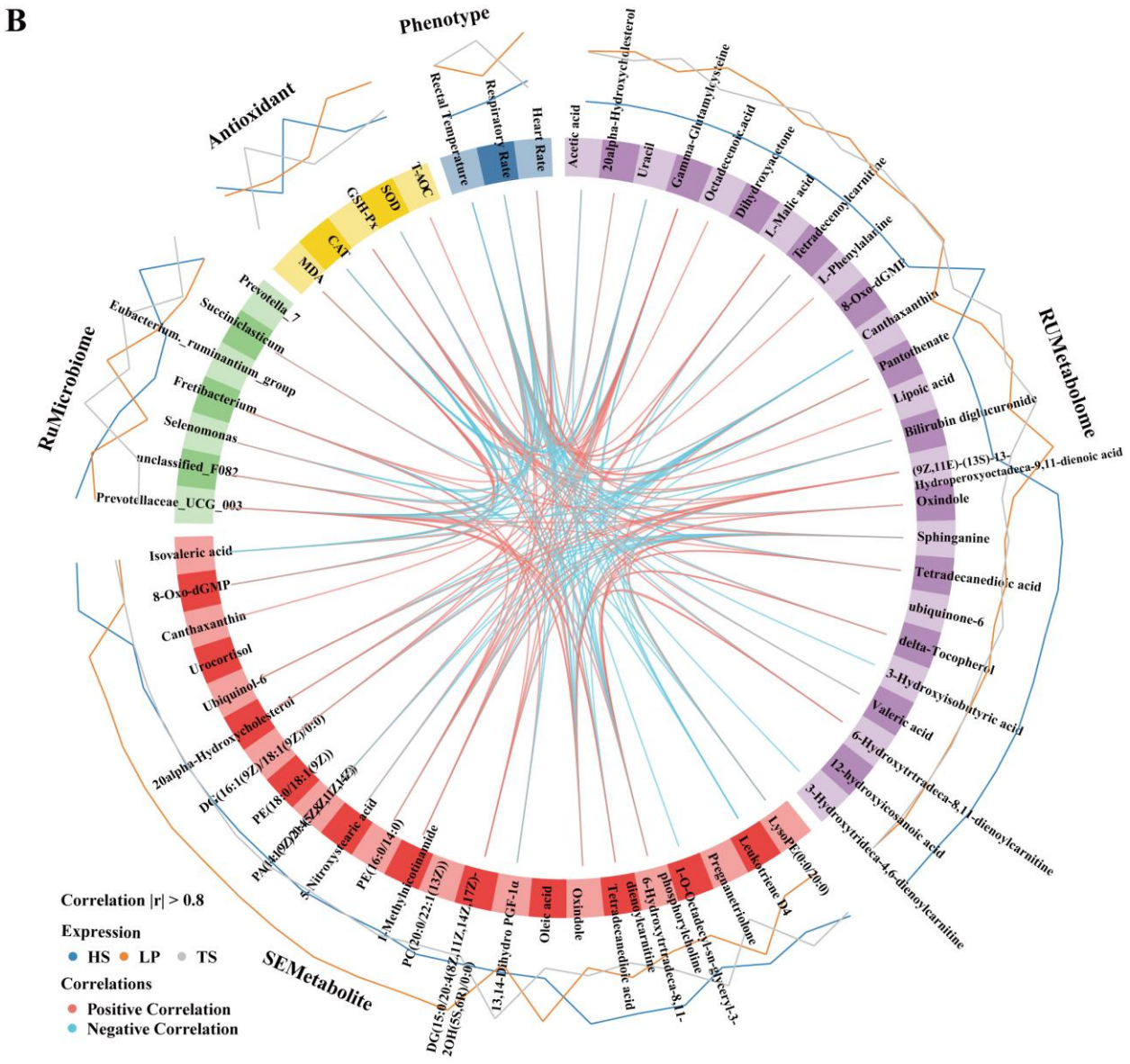

B
Phenotype
Antioxidant
RuMicrobiome
RUMetabolome
SEMetabolite
Rectal Temperature
Respiratory Rate
Heart Rate
T-AOC
SOD
GSH-Px
CAT
MDA
Prevotella_7
Succiniclasticum
Eubacterium_ruminantium_group
Fretibacterium
Selenomonas
unclassified_F082
Prevotellaceae_UCG_003
Isovaleric acid
8-Oxo-dGMP
Canthaxanthin
Urocortisol
Ubiquinol-6
20alpha-Hydroxycholesterol
DG(16:1(9Z)/18:1(9Z)/0:0)
PE(18:0/18:1(9Z))
PA(8:0/20:4(5Z,8Z,11Z,14Z))
5-Nitroxystearic acid
PE(16:0/14:0)
1-Methylnicotinamide
PC(20:0/22:1(13Z))
PGF-1α
Oleic acid
Oxindole
Tetradecanedioic acid
6-Hydroxytridecanoylcarnitine
1-O-Octadecyl-sn-glyceryl-3-phosphorylcholine
Pregnanetriolone
Leukotriene D4
LysoPE(0:0/20:0)
3-Hydroxytrideca-4,6-dienoylcarnitine
12-hydroxyicosanoic acid
6-Hydroxytrtradeca-8,11-dienoylcarnitine
Valeric acid
3-Hydroxyisobutyric acid
delta-Tocopherol
ubiquinone-6
Tetradecanedioic acid
Sphinganine
Oxindole
(9Z,11E)-(13S)-13-Hydroperoxyoctadeca-9,11-dienoic acid
Bilirubin diglucuronide
Lipoic acid
Pantothenate
Canthaxanthin
8-Oxo-dGMP
L-Phenylalanine
Tetradecenoylcarnitine
L-Malic acid
Dihydroxyacetone
Octadecenoic acid
Gamma-Glutamylcysteine
Uracil
20alpha-Hydroxycholesterol
Acetic acid
13,14-Dihydro
DG(15:0/20:4(8Z,11Z,14Z,17Z)-2OH(5S,6R)/0:0)
Correlation |r| > 0.8
Expression
HS
LP
TS
Correlations
Positive Correlation
Negative Correlation

Fig. 7 Multi-omics analysis of different sheep breeds under HS. (A) Correlation analysis and sample distribution analysis of Multi-omics data from different breeds under HS. (B) Multi-omics association network diagram of different breeds under HS.

Discussion

THI is an important indicator of environmental thermal comfort and is widely used to assess the severity of HS in livestock [16]. Studies have found that under HS conditions, RT and RR significantly increase in dairy goats [17]. In the present study, the THI reached 85.23 during the HS period, which exceeded 82 and indicating that the experimental animals were clearly living in a highly heat-stress environment. Under high-temperature conditions, sheep increase their RR to enhance evaporative heat loss through the respiratory tract, thereby facilitating the heat dissipation [18]. Accordingly, the HR, RR, and RT of three sheep breeds markedly increased during HS period. Notably, these physiological parameters of Hus were significantly lower than those of LP and TS, suggesting that HuS exhibited a greater ability for thermoregulation and heat tolerance, while LP and TS appeared to be more susceptible to HS. The antioxidant enzyme system in vivo including SOD, CAT, and GSH-Px, which helps scavenging superoxide anion radicals. Free radical production and clearance remain balanced under physiological conditions to protecting tissues from oxidative stress and damage [19]. T-AOC is widely used to represent the body's antioxidant status, while MDA serves as a comprehensive indicator of lipid peroxidation [20]. During HS, the increase of body temperature affects the activity of metabolic enzymes in the body, accelerates metabolism, and induces the production of free radicals, thereby disrupting the body's antioxidant capacity [21, 22]. Previous research has shown that HS can reduce the activities of SOD and GSH-Px in cows' serum, thereby increasing oxidative damage [23]. Similarly, Aderao et al. found that depressed activities of SOD and GSH-Px and the accumulation of MDA content in the serum of heat-stressed goats, indicating a decrease in their antioxidant capacity [24].

Consistent with previous studies, our research revealed that under HS condition, the activities of SOD in three sheep breeds, as well as the activities GSH-Px in HuS, and CAT in LP and TS, significantly increasedd, while the CAT activity in HuS significantly decreased. Furthermore, the T-AOC levels in three sheep breeds also decreased, and the MDA levels significantly decreased in HuS and TS. These findings indicate that the three breeds exhibited different antioxidant respons processes to HS rather than a uniform pattern of oxidative adaptation. HuS exhibited a relatively coordinated heat-adaptation phenotype. This profile featured a milder physiological heat load, paired with higher GSH-Px activity and lower MDA levels, which supported enhanced protection against oxidative damage. By contrast, LP rely heavily on SOD and CAT contered antioxidant defenses, alongside physiological adjustments to thermal stress. TS bore a heavier thermal burden, as evidenced by elevated body temperature and respiratory rate. Even so, the increased CAT activity and diminished MDA levels pointed to improved capacity to limit oxidative damage.

The rumen microbiota play a central role in nutrient metabolism and host energy supply. Environmental temperature can alter the activity and fuction of ruminal microbes thereby modulating nutrient digestion and metabolism in sheep [25, 26]. In this study, Firmicutes and Bacteroidota dominanted the ruminal bacterial phyla across the three sheep breeds, reflecting their important roles to ruminal carbohydrate degradation, protein metabolism, and energy production [27]. *Fibrobacter* is a major cellulolytic bacterium involved in plant cell-wall degradation [28]. *Prevotella* contributes to the breakdown of hemicellulose, pectin, and protein [29]. *Succiniclasticum* converts succinate to propionate [30]. *Selenomonas* can utilise fermentation intermediates to support propionate synthesis [28]. HS induced divergent, breed-specific shifts at the genus level, pointed to interbreed variation in microbial sensitivity and adaptive plasticity. LP showed marked reduction in *Butyrivibrio*, *Saccharofermentans*, and *Ruminococcus*. Such concurrent declines declines imply more severe

disturbance to fiber degradation and succinate-propionate metabolism in LP. By comparison, HuS displayed evevated *Prevotella* and *Selenomonas*, alongside lower abundances of *Ruminococcus*, *Butyrivibrio*, and *Saccharofermentans*. Zhao et al. [10] and Li et al. [31] reported analogous patterns in dairy cows and dairy goats: heat stress increased lactate-producing and starch-degrading microbes while suppressing fibre-degrading taxa. These findings illustrate that HS remodels the rumen microbiota toward a community specialised in soluble carbohydrate fermentation, at the expense of fiber-associated populations. In TS, the enrichment of *Selenomonas*, *Veillonellaceae_UCG_001*, and *Quinella*, alongside lower Succiniclasticum abundance, signals a rearrangement of fermentation pathways. Notably, *Quinella* possesses metabolic capacities linked to lactate and propionate synthesis, which may be associated with reduced methane production [32]. Such microbil responses to environmental rshifts align with previous studies, confirming that rumen microbiota can undergo substantial restructuring in response to changes in temperature, altitude, season, and forage conditions. He et al. [13] found that relocation of HuS from humid low-altitude to arid high-altitude environments enriched *Prevotella* and upregulated representative rumen metabolites (e.g., 4,6-isocanedione and methanesulfonic acid), while the abundance of S*elenomonas* declined. Likewise, Wang et al. [33] found that high-altitude adaptation in TS increased *Bacteroidota* and *Neocallimastigomycota* and altered rumen metabolites involved in amino acid and lipid metabolism. These findings support the view that the rumen microbiome represents a plastic component of host environmental adaptation. Taken together, the present results suggest distinct microbial response strategies among the three breeds: LP showed greater sensitivity of fiber-degrading and succinate-propionate-associated taxa, whereas HuS and TS exhibited greater enrichment of fermentation-associated bacteria, particularly *Selenomonas*. Such breed-specific microbial plasticity may influence ruminal fermentation and metabolite production and thereby contribute to systemic heat adaptation.

Under CON, the ruminal and serum metabolic profiles of the three breeds were relatively similar, whereas under HS they diverged markedly. This suggests that high temperatures not only disrupt metabolic homeostasis but also induce distinctly different metabolic regulations in the rumen and systemic circulation. These differences may be attributed to variations in nutrient allocation and energy utilization among the different breeds [15, 34]. In this study, changes in the rumen metabolic profile were primarily associated with alterations in carbohydrates, amino acids, fatty acids, vitamins, and metabolites related to microbial fermentation. Serum metabolites were more closely associated with amino acid, lipid, bile acid, steroid, and endocrine metabolism. Importantly, metabolites shared between the rumen and serum revealed coordinated yet compartment-specific metabolic responses. Lipid-related metabolites, particularly 6-hydroxytrtradeca-8,11-dienoylcarnitine and tetradecanedioic acid, showed marked breed-dependent changes in both compartments, suggesting remodeling of fatty acid utilization and oxidative metabolism during HS. Feng et al. found that metabolic changes under HS are closely related to arachidonic acid metabolism, fatty acid biosynthesis, and energy metabolism, and suggested that rumen microorganisms may participate in the regulation of lipid metabolism through fatty acyl metabolism [35]. Acylcarnitines are involved in mitochondrial fatty acid transport and oxidation, whereas dicarboxylic acids can be generated through fatty acid ω-oxidation, indicating that alternative routes of lipid oxidation may contribute to metabolic adaptation [36, 37]. 8-Oxo-dGMP was markedly elevated in the serum of HuS but showed higher ruminal abundance in LP and TS, indicating differential regulation of oxidative nucleotide metabolism between the systemic circulation and rumen. Together with the relatively lower physiological heat load and enhanced GSH-Px activity in HuS, these findings suggest a coordinated systemic metabolic and antioxidant response. In contrast, the pronounced ruminal changes in Oxindole, lipid-related metabolites, and canthaxanthin in LP point to greater involvement of rumen metabolic and redox remodeling. Xu et al. found that under HS, there

were significant differences in serum metabolism between HuS and Dorper sheep, primarily involving protein, carbohydrate, and lipid metabolism. In addition, HuS exhibited higher T-AOC and GSH-Px levels[14]. Overall, the shared rumen-serum metabolite profiles indicate that heat adaptation involves coordinated metabolic responses across compartments rather than an isolated change in either the rumen or circulation. The distinct distribution of lipid- and nucleotide-related metabolites further suggests that different breeds may preferentially engage ruminal or systemic metabolic pathways to cope with increased energetic and oxidative demands. Thus, breed-specific heat adaptation may involve compartment-specific reprogramming of lipid, nucleotide, and redox metabolism that contributes to mitigating heat-induced metabolic disturbance.

Under HS, metabolic reprogramming and disruptions in redox homeostasis are considered key factors affecting livestock adaptability. Changes in ruminal metabolism may promote the host's thermal adaptation by regulating systemic oxidative status. In this study, L-malic acid, Gamma-Glutamylcysteine, and GSH-Px were closely correlated. L-malic acid is a key intermediate of the tricarboxylic acid (TCA) cycle and plays an essential role in carbon flux redistribution and energy metabolism [38]. whereas Gamma-Glutamylcysteine is the rate-limiting precursor for glutathione synthesis and contributes to maintaining cellular antioxidant capacity [39]. In the present study, L-malic acid showed strong negative correlations with serum oxidative stress-related metabolites, including Oxindole and 8-Oxo-dGMP. Similarly, Gamma-Glutamylcysteine was negatively correlated with Oxindole, 8-Oxo-dGMP, and 6-Hydroxytrtradeca-8,11-dienoylcarnitine, a lipid oxidation-associated metabolite. These findings suggest that enhanced rumen malate metabolism and increased availability of glutathione precursors may be associated with reduced systemic oxidative damage during HS. Heat exposure promotes excessive production of reactive oxygen species (ROS) and activates antioxidant defenses to maintain redox homeostasis [40]. Therefore, the maintenance of TCA

cycle activity and glutathione synthesis may provide metabolic support for preserving redox balance under thermal challenge. Studies have found that GSH-Px activity was strongly positively correlated with 3-Hydroxyisobutyric acid, a metabolite associated with branched-chain amino acid (BCAA) catabolism [41]. This suggests that antioxidant capacity is closely linked to amino acid oxidation and energy metabolism. Oxindole and 8-Oxo-dGMP showed significant correlations with RT and RR. 8-Oxo-dGMP is a representative marker of DNA oxidative damage [42]. This suggests that thermal stress is closely associated with oxidative damage. Moreover, 8-Oxo-dGMP was negatively correlated with several rumen metabolites, including L-malic acid, acetic acid, dihydroxyacetone, and octadecenoic acid, but positively correlated with 3-Hydroxyisobutyric acid, δ-tocopherol, and sphinganine. These relationships suggest that 8-Oxo-dGMP may integrate information from energy metabolism, lipid metabolism, and antioxidant compensation pathways. Similarly, serum Oxindole was positively correlated with rumen Oxindole but negatively associated with rumen L-malic acid, lipoic acid, and Gamma-Glutamylcysteine, suggesting that indole-related metabolites derived from tryptophan metabolism may participate in rumen-host communication and reflect systemic oxidative status. Rumen microorganisms may serve as important mediators regulating this metabolic network. In this study, *Fretibacterium* showed positive correlations with L-malic acid and Gamma-Glutamylcysteine, but a negative correlation with Oxindole. This pattern suggests that *Fretibacterium* may be associated with the accumulation of beneficial metabolic intermediates and reduced oxidative stress. *Fretibacterium* has been shown to be involved in the host's protein metabolism, amino acid transformation, and fermentation regulation [43]. In addition, Succiniclasticum was negatively correlated with acetic acid but positively correlated with valeric acid and tetradecanedioic acid, indicating alterations in succinate utilization pathways and volatile fatty acid profiles under HS. Volatile fatty acids are the primary energy source for ruminants, and changes in fermentation patterns

may affect the host's energy supply and oxidative metabolism [44, 45]. Collectively, rumen microorganisms mitigate oxidative damage caused by HS by regulating the production of metabolites, thereby influencing the host's energy metabolism and antioxidant capacity. These interactions among microorganisms, metabolism, and physiology exhibit species-specific differences, which may reflect the unique adaptive strategies that sheep have developed through long-term environmental selection.

Conclusion

Under HS, HR, RR, and RT increased in all three varieties. HuS exhibited the lowest physiological stress indicators and the best heat tolerance; TS had the highest RR and respiratory duration and was the most heat-sensitive; LP had the highest HR, but its RR was lower than that of TS. The breeds also differed in their antioxidant responses. HuS showed higher glutathione peroxidase activity, LP greater superoxide dismutase activity, and TS lower malondialdehyde levels. HS altered key rumen microbes, including *Prevotella*, *Selenomonas*, and *Succiniclasticum*. In addition, HS reshaped the metabolic profiles of the rumen and serum. L-malic acid and Gamma-Glutamylcysteine were closely associated with metabolites related to oxidative stress, while 8-oxo-dGMP was closely associated with RT and RR. Collectively, these findings indicate that breed-specific heat adaptation involves coordinated changes in thermoregulation, antioxidant defense, rumen microbiota, and systemic metabolism. HuS showed relatively greater heat tolerance, whereas TS were more heat-sensitive and LP exhibited enhanced antioxidant defense despite a higher physiological heat load.

Declarations

Abbreviations

HS	heat stress
CON	non-heat stress
HuS	Hu sheep

LP	Lanping black-boned sheep
TS	Tibetan sheep
THI	temperature-humidity index
GSH-Px	Glutathione peroxidase
T-AOC	total antioxidant capacity
SOD	superoxide dismutase
CAT	catalase
MDA	malondialdehyde
ROS	reactive oxygen species
RT	rectal temperature
RR	respiratory rate
HR	heart rate
VFA	volatile fatty acid
LEfSe	Linear discriminant analysis effect size
QC	quality control
LC-MS/MS	Liquid chromatography tandem mass spectrometry
VIP	variable importance in projection
FC	fold change
OPLS-DA	Orthogonal partial least-squares discriminant analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
ANOVA	one-way analysis of variance
FDR	false discovery rate

Ethics approval and consent to participate

This experimental protocol was approved by the Institutional Animal Care and Use Committee of Anhui Agricultural University (protocol number: KJLL2023021). Informed consent was obtained from

all owners of the animals involved in this study before sample collection.

The authors declared that all animal management and experimental procedures were performed in compliance with the Guidelines for Laboratory Animals established by the Ministry of Science and Technology of China.

Consent for publication

Not Applicable.

Clarification on how the animal was euthanized

Not Applicable.

Availability of data and materials

Data is provided within the manuscript or supplementary information files.

Supplementary Information

Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3

Supplementary Table 1

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability

The datasets generated during the current study are available in the Sequence Read Archive National Center for Biotechnology Information (NCBI) repository, PRJNA1557037.

Authors' contributions

XC, CR, QW, and YH: designed the study and provided the sources of all the samples, and wrote the manuscript; TJ and WT: writing original draft, collation and analysis of experimental data, and plotting using software; XG, JH, NC, JT, RP, OP, SW and YC: collected the data and helped complete the experiments; JC: provision of test sites; XC and ZZ: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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