Animal Microbiome

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Metagenomic analysis reveals microbial drivers of heat resistance in dairy cattle

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Abstract

Heat stress poses a significant challenge to dairy cattle, leading to adverse physiological effects, reduced milk yield, impaired reproduction performance and economic losses. This study investigates the role of the rumen microbiome in mediating heat resistance in dairy cows. Using the entropy-weighted TOPSIS method, we classified 120 dairy cows into heat-resistant (HR) and heat-sensitive (HS) groups based on physiological and biochemical markers, including rectal temperature (RT), respiratory rate (RR), salivation index (SI) and serum levels of potassium ion (K^+), heat shock protein 70 (HSP70) and cortisol. Metagenomic sequencing of rumen fluid samples revealed distinct microbial compositions and functional profiles between the two groups. HR cows exhibited a more cohesive and functionally stable microbiome, dominated by taxa such as *Ruminococcus flavefaciens* and *Succiniclasticum*, which are key players in fiber degradation and short-chain fatty acid production. Functional analysis highlighted the enrichment of the pentose phosphate pathway (PPP) in HR cows, suggesting a metabolic adaptation that enhances oxidative stress management. In contrast, HS cows showed increased activity in the tricarboxylic acid (TCA) cycle, pyruvate metabolism and other energy-intensive pathways, indicating a higher metabolic burden under heat stress. These findings underscore the critical role of the rumen microbiome in modulating heat resistance and suggest potential microbiome-based strategies for improving dairy cattle resilience to climate change.

Keywords Rumen microbiome, Heat resistance, Dairy cattle, *Ruminococcus flavefaciens*, Pentose phosphate pathway

Introduction

Global climate change, driven by increasing greenhouse gas emissions, presents a significant threat to livestock production, with dairy cattle being particularly vulnerable to heat stress [1, 2]. This stress leads to substantial physiological and metabolic disruptions, including reduced feed intake, lower milk yield, impaired reproductive performance, and increased susceptibility to diseases such as mastitis and metabolic disorders [3–5]. These detrimental effects not only compromise animal health but also impose substantial economic burdens on the dairy industry [6].

Despite extensive research on the physiological consequences of heat stress, the biological mechanisms underlying heat resistance in dairy cattle remain inadequately explored [7]. Traditional studies have primarily focused on phenotypic indicators like rectal temperature (RT), respiratory rate (RR), salivation index (SI) and milk production (MP) to assess heat stress [8]. However, these metrics, while informative, do not capture the intricate, multi-layered biological processes that distinguish heat-resistant animals from their heat-sensitive counterparts. This knowledge gap

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has hindered the development of effective strategies to enhance heat resistance in dairy cattle, a necessity that is becoming increasingly urgent as global temperatures rise [9].

A critical element of dairy cattle's biological response to heat stress is the rumen microbiome, which plays a pivotal role in overall health and productivity [10]. The rumen, a specialized fermentation chamber, hosts a diverse consortium of microorganisms essential for the digestion of complex plant materials [11, 12]. The balance and functionality of this microbial community are crucial for maintaining digestive efficiency and overall metabolic health, particularly under stress conditions like heat stress [13]. However, the specific relationship between heat stress and the rumen microbiome is still not fully understood. It is well-documented that heat stress can disrupt the equilibrium of the rumen microbiota, leading to shifts in fermentation patterns, nutrient absorption, and microbial community structure [14–16]. For example, heat stress has been linked to a reduction in key fiber-degrading bacteria such as *Streptococcus*, one possible reason is that the *Fibrobacterales* order and families within it exhibit higher heat resistance compared to other rumen bacteria [17]. Studies have shown that changes in microbial activity and circulating cytokine levels trigger physiological and immune responses in animals exposed to heat stress, supporting the brain-gut axis concept in dairy cows [18]. It is worth noting that the connection between the rumen microbiome and heat stress may be indirect, as heat stress can lead to changes in several factors, such as reduced dry matter intake, selective consumption of specific feed components, decreased rumination time, and reduced salivary bicarbonate infusion into the rumen [19]. All of these are associated with the gut microbiome and metabolism.

Recent advancements in high-throughput sequencing technologies, particularly metagenomics, have provided unprecedented insights into the composition and function of the rumen microbiome [20]. Metagenomic analyses have revealed that certain microbial taxa, including specific strains of *Ruminococcus* and *Bacteroides*, are more prevalent in heat-resistant cattle, suggesting a protective role for these microorganisms against heat stress [21, 22]. Despite these technological advancements, there is still a lack of systematic studies exploring the intricate interactions between heat stress, rumen microbiota, and heat resistance in dairy cattle. Most existing research has focused on the effects of diet and environmental factors on the rumen microbiome, with relatively few studies investigating the impact of heat stress [23]. Furthermore, the potential for identifying microbial biomarkers of heat resistance—specific microorganisms or metabolic pathways that could serve as predictive indicators or therapeutic targets—remains largely untapped.

This study aims to address these gaps by conducting a comprehensive analysis of the rumen microbiome in dairy cattle subjected to heat stress. Utilizing the power of metagenomic sequencing, we will characterize the shifts in microbial composition and function associated with heat stress and identify specific microbial taxa linked to enhanced heat resistance. By correlating these microbial changes with physiological indicators of heat stress, such as RT, RR, and SI, we expect to elucidate the microbial mechanisms that contribute to heat resistance. The insights gained from this study could pave the way for novel microbiome-based strategies to improve the resilience of dairy cattle to heat stress, thereby enhancing both animal welfare and production efficiency.

Materials and methods

Experimental design and sampling

This experiment was conducted in Weihang Farm in Suqian, Jiangsu province for 50 days from mid-July to the end of September 2023, to evaluate the physiological and microbial responses of dairy cows under heat stress conditions. A total of 120 high-yielding Chinese Holstein cows were selected as subjects for this study (parity: 2.21 ± 0.79 years; days in milk: 206.71 ± 54.29 days; average milk yield: 34.21 ± 18.09 kg; Mean ± SEM). To mitigate the effects of heat stress, the cows were housed in a well-ventilated barn equipped with fans and an automatic sensor-based spraying system. Additionally, a 30-min cooling spray was applied before each milking session to lower the cows'body temperature and enhance comfort during the high-temperature period. These measures aimed to reduce thermal load while maintaining the cows'welfare and productivity.

After more than two months of heat stress measurements, the entropy-weighted TOPSIS method was applied to evaluate physiological indicators such as RT, RR, and SI, as well as blood biochemical indicators including potassium ion (K^+) concentration, heat shock protein 70 (HSP70), and cortisol levels. Based on this weighted analysis, heat-sensitive (HS) cows(n = 6) and six heat-resistant (HR) cows(n = 6) were ultimately selected. To minimize the influence of environmental factors, the cows were housed in the same barn and fed a total mixed ration (TMR) three times daily at 6:30, 14:30, and 20:00, with unrestricted access to feed and water.

Heat stress monitoring

To directly reflect the environmental heat stress level, the Temperature-Humidity Index (THI), a widely recognized metric for assessing heat stress, was calculated using the formula [24]:

$$THI = 0.81 \times T + 0.99 \times T - 14.3 \times R + 46.3$$

where T represents the ambient temperature in degrees Celsius (°C), and R represents relative humidity in percentage (%). Two temperature and humidity recorders were strategically placed above the bedding on both sides of the barn, capturing data every five minutes throughout the experimental period. The average THI over the measurement period was used to represent daily THI.

Physiological and performance measurements

The AfiLab system (Afimilk, Kibbutz Afikim, Israel) is a real-time individual cow milk analyzer installed in each milking stall. Lactating cows are milked daily at 05:00, 13:00, and 19:30 in a rotary herringbone milking parlor equipped with this system, using near-infrared spectroscopy technology to measure individual daily milk production (kg) and pre-milking rumination frequency (RF) and feeding time (FT).

To evaluate heat resistance, key physiological indicators—RT, RR, and SI—were measured daily between 13:00 and 15:00, a period of peak daytime heat. RT was recorded using a calibrated electronic thermometer; RR was determined by counting chest movements over 30 s; SI was assessed by visual inspection of the cows'mouth and nose, the detailed scoring rules are shown in Table 1. These measurements were taken with a high sampling intensity to capture daily fluctuations and assess individual heat resistance. Additionally, milk yield was recorded daily, and percentage changes were calculated by comparing values between the non-thermoneutral (NTN; May to June) and thermoneutral (TN; July to August) periods.

Sample collection

To evaluate heat resistance in dairy cows via blood biomarkers, blood samples (5 mL) were obtained from the coccygeal vein utilizing sterile disposable needles and vacutainers on day 25 of the trait measurement trial. The samples were permitted to coagulate at ambient temperature for 20 min prior to centrifugation at 3500 r/min for 5 min. Subsequently, serum was then aliquoted into 2 mL cryovials and stored at −80 °C for future biochemical analysis. Rumen fluid samples were collected on day 50 after categorizing the cows into HR and HS groups using a rumen tube. The samples were then filtered through four layers of sterile gauze, aliquoted into 10 mL cryovials, and stored at −80 °C for subsequent metagenomic sequencing.

Determination of serum biochemical markers

K^+ concentration was measured using an IMS- 972 electrolyte analyzer (Xilaiheng Medical Electronics Co., Ltd, Shenzhen, China) [25]. The levels of HSP70 and cortisol in serum were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Jiangsu Jingmei Biotechnology Co., Ltd, Yancheng, China).

Selection of heat-resistant cows using entropy-weighted TOPSIS

The Technique for Order of Preference by Similarity to Ideal Solution (TOPSIS) is a multi-criteria decision-making algorithm that ranks options based on their proximity to an ideal solution. In this study, TOPSIS was combined with the entropy weight method to evaluate heat resistance in dairy cows. The entropy weight method was first used to assign weights to various heat resistance indicators, such as RT, RR, SI, changes in milk yield, K^+ concentration, cortisol levels, and HSP70 levels, for a group of 120 Holstein cows. These weights reflect the relative importance of each indicator in assessing heat resistance.

After determining the weights, the data for each cow were multiplied by these weights to create a weighted dataset. The TOPSIS method was then applied to this dataset to rank and evaluate the heat resistance of each cow. Before this analysis, the data underwent thorough cleaning, including removing outliers, imputing missing values, and normalizing the data. The cleaned and processed data were compiled into an Excel spreadsheet, where the final heat resistance scores were calculated using the entropy-weighted TOPSIS method. This approach allowed for a nuanced and comprehensive assessment of heat resistance across the herd.

(1) Data normalization:

For positive indicators:

Table 1 The standard of salivation index of lactating cows

Salivation index	Salivation condition	Snout condition
1	Almost no runny nose or saliva	Nose wet, chin clean
2	A small amount of saliva flows out, hanging in a filamentary or dripping water	Chin is slightly moist, with a little forage on it
3	Drooling or snot flowing down in streams, or even gasping for breath	The chin was moist and heavily fed

$$y_{ij} = \frac{x_{ij} - MINx_{ij}}{MAXx_{ij} - MINx_{ij}}$$

For negative indicators:

$$y_{ij} = \frac{MAXx_{ij} - x_{ij}}{MAXx_{ij} - MINx_{ij}}$$

(2) Determining the proportion p_{ij} of the i^{th} evaluation object in the j^{th} indicator:

$$p_{ij} = \frac{y_{ij}}{\sum_{i=1}^{m} y_{ij}}$$

(3) Calculating the entropy value e_j for the j^{th} indicator:

$$e_j = -\frac{1}{\ln m} \sum_{i=1}^{m} p_{ij} \ln p_{ij}, 0 \le e_j \le 1$$

where p_{ij} is the proportion of the i^{th} sample under the j^{th} indicator; y_{ij} is the normalized value of the i^{th} sample under the j^{th} indicator; e_j is the entropy value of the j^{th} indicator; and m is the number of samples.

(4) Determining the final entropy weight values w_j of the j^{th} indicator:

$$w_j = \frac{g_j}{\sum_{j=1}^{m} g_j}, \text{where } g_j = 1 - e_j$$

where $g_j = 1 - e_j$ represents the degree of dispersion for the j^{th} indicator, and w_j is the weight of the j^{th} indicator derived using the entropy method. The weights satisfy the conditions $\sum_{j=1}^{m} w_j = 1, w_j \ge 0, j \in N$; and g_j reflects the degree of dispersion in the evaluation data for the j^{th} indicator.

(5) Multiplying the weights by the normalized decision matrix to obtain the weighted decision matrix V_i:

$$V_i = \sum_{j=1}^{m} \left(w_j * y_{ij}\right) (i = 1, 2, \ldots, m)$$

V_i represents the comprehensive heat resistance score of the i^{th} sample.

(6) Determining the positive and negative ideal solutions and calculating the distance from each sample to the positive and negative ideal solutions:

$$D_i^+ = \sqrt{\sum_{j=1}^{m} \left(z_{ij} - z_j^+\right)^2}, D_i^- = \sqrt{\sum_{j=1}^{m} \left(z_{ij} - z_j^-\right)^2}, (i = 1, \ldots, n)$$

Z^+ represents the positive ideal solution where each indicator reaches the best value within the sample set, while Z^- represents the negative ideal solution where each indicator reaches the worst value within the sample set.

(7) Calculating the Relative Closeness C_i of Each Sample to the Heat Resistance Performance:

$$C_i = \frac{C_i^-}{C_i^+ + C_i^-}$$

Values of C_i range from 0 to 1, with values closer to 1 indicating stronger heat resistance.

Using this methodology, the top 5% of cows (n =6) were identified as HR, and the bottom 5% (n =6) were classified as HS.

DNA extraction and metagenomic sequencing

Rumen fluid samples from HR and HS cows were subjected to comprehensive metagenomic sequencing. The DNA extraction process was optimized to ensure high-quality genomic material for downstream analyses. Specifically, genomic DNA was extracted using the CTAB method, and DNA concentration and purity were measured with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA) [26]. Genomic DNA integrity was confirmed via 1% agarose gel electrophoresis, and samples were stored at − 80°C until further use.

Metagenomics was employed to analyze the distribution of bacterial species and functional genes. Metagenomic libraries were prepared using the VAHTS® Universal Plus DNA Library Prep Kit (Vazyme, Nanjing, China) [27], selecting fragment sizes with an average length of 420–580 bp. The library construction process involves digesting genomic DNA, adding ligation reagents, followed by purification and washing with magnetic beads. PCR amplification is then performed to generate products (adpter3 ="AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC"; adpter5 ="AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT"), which underwent two rounds of magnetic bead selection and ethanol washing, resulting in high-quality DNA fragments for subsequent analysis. Libraries were sequenced on the Illumina NovaSeq 6000 platform [28], producing high-quality reads for analysis.

Bioinformatics and statistical analysis

Based on the repetitive relationships between sequences, Fastp, MEGAHIT and MMseqs2 software were used to assemble, filter, and remove chimeras from the raw data [29–31]. Non-redundant sequences were subjected to species classification analysis at 97% similarity. Species annotation was conducted by aligning non-redundant genes with sequences in the non-redundant protein database (Nr) [32]. The microbial community composition of

each sample was analyzed at the levels of kingdom, phylum, class, order, family, genus, and species.

Alpha diversity analysis and Partial Least Squares Discriminant Analysis (PLS-DA) between groups were performed using R v3.1.1 (picante, v1.8.2; mixOmics, v6.3.2) [33, 34]. The Alpha diversity indices included ACE, Chao1, Simpson, and Shannon indices. Functional annotations were conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to identify metabolic pathways enriched in HR and HS cows [35]. Carbohydrate-active enzymes (CAZymes) were identified using HMMER software [36, 37], and their roles in rumen fermentation were analyzed. Random Forest and Linear Discriminant Analysis Effect Size (LEfSe) analyses were conducted using R v3.1.1 (random Forest v4.6–10) and Python 2 (lefse v20171228) to identify key microbial taxa associated with heat resistance [38, 39]. Differential microbial species were screened at the species level using thresholds of $P < 0.05$ and $|\log_2 FC| \geq 1.5$.

The variables were standardized, and a mixed linear model was conducted using SPSS 20.0. Groups (HR and HS) and time points (TN and NTN periods) were set as fixed effects, while days in milk (DIM) and parity were included as covariates. Individual cows were treated as random effects to account for variability from repeated measurements. General linear model analysis was performed using Origin 2021, with a 95% confidence interval and a significance level of $P < 0.05$. Pearson's correlation coefficient was used to evaluate correlations between variables, while Spearman's rank correlation analysis was applied for non-normally distributed data. Differences between groups were compared using the Wilcoxon rank-sum test, and multiple comparisons were adjusted using the false discovery rate (FDR) correction.

Results

Identification of heat-resistant cows using entropy-weighted TOPSIS

During the trial period, the daily average THI was 81.1 ±3.17, with the minimum THI value consistently exceeding 68 (Fig. 1A) [40]. Cows transition from a thermoneutral state into a heat stress response. The high-temperature environment during NTN period resulted in significantly lower average milk yield and RF compared to the TN period ($P < 0.001$). However, there was no significant difference in FT between the two groups (386.1 min/d vs. 382.6 min/d, $P = 0.693$; Fig. 1B). Physiological parameters indicated that RT, SI, and RR were positively correlated with increasing THI, with significant correlations observed for SI and RR (*Pearson's r* > 0.5, Fig. 1C).

Using the entropy-weighted TOPSIS method, we classified 120 dairy cows based on physiological and biochemical markers of heat stress, including RT, RR, SI, change in milk yield and serum levels of K^+, HSP70 and cortisol. This approach effectively identified the top 5% of cows as HR and the bottom 5% as HS (Tables 2 and 3). HR cows exhibited significantly lower salivation index compared to HS cows ($P < 0.05$). HR cows also exhibited a lower respiratory rate. However, this difference did not reach statistical significance. These findings suggest a more efficient thermoregulatory response under heat stress. Additionally, HR cows showed elevated levels of HSP70 and cortisol ($P < 0.01$), biomarkers associated with a robust stress response (Fig. 2).

Composition and diversity of the rumen microbiome

Metagenomic sequencing of rumen samples from HR and HS cows generated over 156.3 billion raw read pairs. After stringent quality filtering, including the removal of low-quality reads and host DNA contamination, 9.93 billion high-quality read pairs remained, averaging 82.7 million pairs per sample with a high-quality read ratio of 96.08%.

The metagenomic analysis identified 23,223 microbial species across several domains, including archaea, bacteria, eukaryotes, fungi, viruses, and metazoans. The rumen microbiome was dominated by four major phyla: *Firmicutes*, *Bacteroidetes*, *Uroviricota*, and *Proteobacteria* (Fig. 3A). *Firmicutes* was the most abundant phylum in both HR and HS cows, accounting for approximately 57.3% of the total microbial population. *Bacteroidetes* (26.9%) was the second most prevalent phylum, followed by *Uroviricota* (5.5%) and *Proteobacteria* (5.2%).

Venn diagram analysis highlighted unique microbial taxa present exclusively in HR or HS cows. HR cows had 15 unique archaeal taxa, 431 bacterial taxa, 448 fungal taxa, and 44 viral taxa, suggesting a more specialized microbial community that could be tailored to support better adaptation to heat stress (Fig. 3B). In contrast, HS cows exhibited a broader range of unique taxa, possibly indicating a more diverse but less stable microbial ecosystem.

The Partial Least Squares Discriminant Analysis (PLS-DA) revealed distinct clustering patterns that corresponded to the heat resistance status of the cows (Fig. 3C), with HR cows displaying a more cohesive and functionally stable microbial community, while HS cows exhibiting a more diverse but potentially less balanced microbiome. Alpha diversity metrics further supported these findings (Fig. 3D). The ACE and Chao1 indices, which measure species richness, were higher in HS cows, indicating greater microbial diversity. However, the Shannon and Simpson indices, which

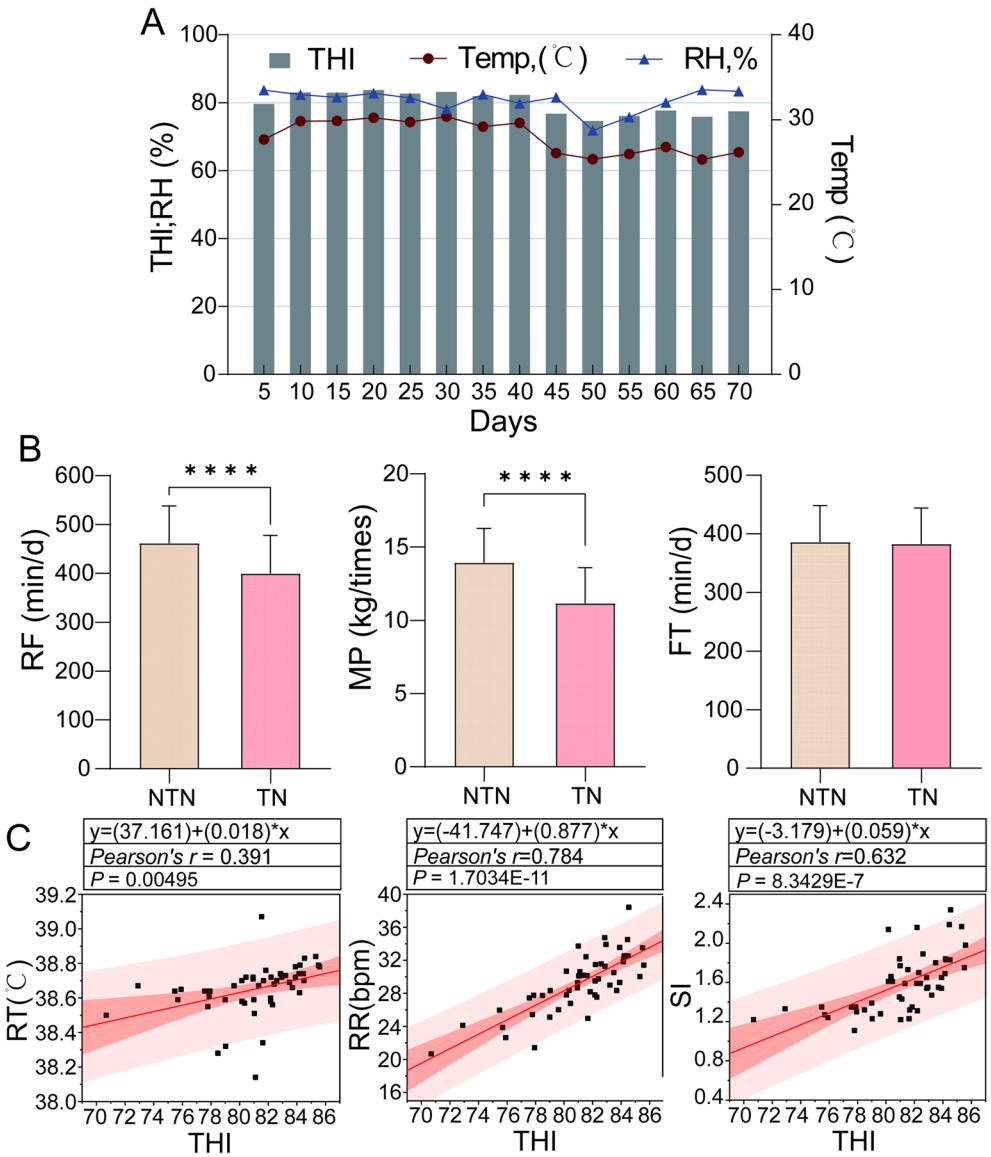

Fig. 1 **A** Average daily values of temperature, humidity, and temperature-humidity index (THI) in the cowshed during the experiment. The x-axis represents the time of heat resistance trait measurement; the y-axis represents environmental temperature, relative humidity, and calculated THI recorded by the temperature-humidity data logger. **B** Changes in average MP, RF, and FT of cows during the thermoneutral condition (TN, May to June) and non-thermoneutral condition (NTN, July to August), MP = milk production; RF = rumination frequency; FT = feeding time. The differences between groups were analyzed using an independent sample t-test, and **** represents $P < 0.0001$. Error bars indicate the standard error of the mean (SEM). **C** The effect of the THI on RT, RR, and SI. RT = rectal temperature; RR = respiration rate; SI = salivation index. The 95% confidence and prediction bands are shown. *Pearson's r* = Pearson correlation coefficient, where the absolute value indicates the strength of the correlation

account for both abundance and evenness, showed that the microbial community in HR cows was more functionally cohesive and stable. This functional stability in HR cows likely contributes to their enhanced resilience under heat stress conditions.

Differential microbial composition between HR and HS cows

The microbial composition between HR and HS cows was different, reflected in *Bacteroidetes* (28% vs 25%) (Fig. 4A). To pinpoint microbial biomarkers that distinguish HR cows from HS cows, we employed Linear Discriminant Analysis Effect Size (LEfSe) (Fig. 4B). This

Table 2 Weight of heat resistance properties calculated by entropy weight method

Entropy-Weighted TOPSIS			
Index	**Entropy value (*e*)**	**Information entropy redundancy (*g*)**	**Weight values (*W*)**
K^+	0.98	0.02	0.13
Cortisol	0.95	0.05	0.29
HSP70	0.95	0.05	0.29
Rectal temperature	0.99	0.01	0.08
Respiratory rate	0.99	0.01	0.08
Salivation index	0.99	0.01	0.05
Changes in milk yield	0.99	0.01	0.08

K^+ represents potassium ion

analysis identified *Ruminococcus flavefaciens* and *Succiniclasticum* as key biomarkers in HR cows, with Linear Discriminant Analysis (LDA) scores exceeding 4.0 ($P < 0.05$). Both taxa are crucial for the fermentation of complex carbohydrates and the production of short-chain fatty acids (SCFAs), which are vital for maintaining energy homeostasis and intestinal health under heat stress conditions. The key bacterium *Firmicutes bacterium CAG- 137*, predicted by the random forest model, is rarely mentioned in the context of stress response (Fig. 4C).

Conversely, in HS cows, species level microorganisms of *Streptococcus* and *Lactobacillus* emerged as prominent taxa (Fig. 4D). These genera are associated with lactate production, which can contribute to an acidogenic rumen environment, potentially exacerbating the effects of heat stress by disrupting rumen pH balance and leading to conditions such as subacute ruminal acidosis (SARA) [41].

Functional annotation and pathway analysis

Functional annotation of the metagenomic data using KEGG pathway analyses revealed significant differences between the rumen microbiomes of HR and HS cows. In HR cows, there was a notable enrichment of the pentose phosphate pathway (PPP), which plays a critical role in generating nicotinamide adenine dinucleotide phosphate (NADPH) [42] (Fig. 5). NADPH is essential for combating oxidative stress, a condition that is exacerbated during heat stress [43]. This enrichment suggests that HR cows are better equipped to manage oxidative stress through enhanced NADPH production, thereby protecting cells from damage.

In contrast, HS cows exhibited a greater abundance of pathways associated with basic energy production, including the tricarboxylic acid (TCA) cycle and pyruvate metabolism. The increased activity in these pathways suggests a higher metabolic cost in HS cows, likely reflecting an attempt to meet the elevated energy demands imposed by heat stress. Additionally, methane metabolism, nitrogen metabolism, and purine metabolism pathways were more pronounced in HS cows, indicating a higher metabolic burden. These pathways, while essential for basic cellular functions, when upregulated, may exacerbate the physiological strain on HS cows under heat stress. The increased activity in these pathways suggests that HS cows are expending significant energy resources to maintain homeostasis under heat stress, which could detract from other vital processes such as growth and milk production.

The analysis of carbohydrate-active enzymes (CAZymes) further highlighted the functional differences between the microbiomes of HR and HS cows. HR cows exhibited a distinct CAZyme profile, with a significant upregulation of glycoside hydrolases (GHs) and carbohydrate-binding modules (CBMs), particularly CBM80 (Fig. 6B). These enzymes are crucial for the degradation of plant cell walls and the efficient extraction of energy from fibrous feedstuffs [44]. The prominence of these enzymes in HR cows supports the notion that their microbiomes are better equipped to maintain energy balance and metabolic health during heat stress. In contrast, the CAZyme profile of HS cows was characterized by a greater abundance of enzymes involved in the breakdown of simpler polysaccharides and sugars, such as GH50, GH109, GH116, GH148, and CBM17. While this enzyme profile may facilitate

Table 3 TOPSIS method was used to calculate the comprehensive scores of HS and HR dairy cows

Heat resistance grouping						
HS	**HS1(16,080)**	**HS2(19,046)**	**HS3(19,235)**	**HS4(19,096)**	**HS5(20,026)**	**HS6(17,006)**
Comprehensive score	0.17	0.18	0.21	0.24	0.24	0.28
HR	HR1(18,152)	HR2(19,018)	HR3(19,038)	HR4(21,043)	HR5(20,002)	HR6(18,160)
Comprehensive score	0.72	0.73	0.75	0.75	0.80	0.81

The comprehensive score represents the heat resistance score of dairy cows, calculated using the entropy-weighted TOPSIS method. The higher the score, the stronger the heat resistance; conversely, the lower the score, the weaker the heat resistance. Samples are labeled as heat-resistant (HR) cows and heat-sensitive (HS) cows followed by a number, with the farm number provided in parentheses

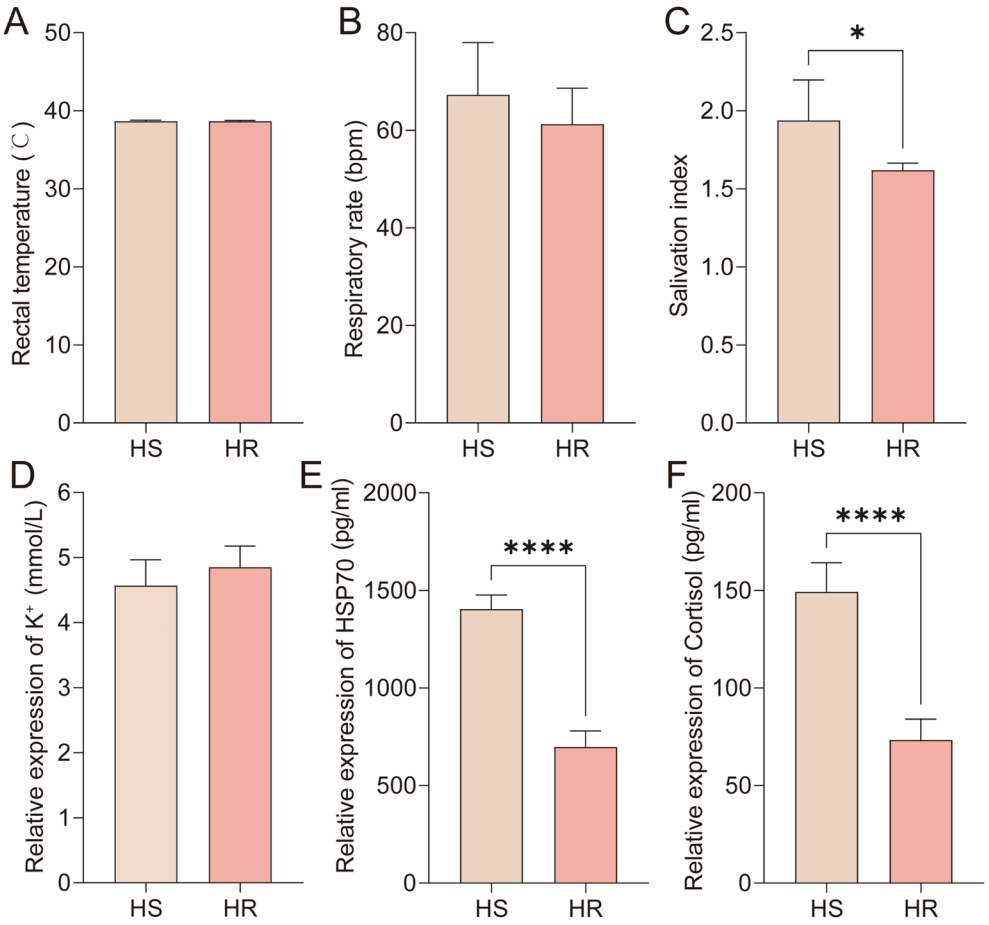

Fig. 2 The analysis of physiological and biochemical markers in HR and HS cows. **A** Rectal temperature, **B** Respiratory rate, **C** Salivation index, **D** serum levels of potassium ion (K^+), (**E**) heat shock protein 70 (HSP70) and (**F**) Cortisol. HR = heat-resistant cows; HS = heat-sensitive cows. * represents $P < 0.05$, significant difference; **** represents $P < 0.0001$, extremely significant difference

rapid fermentation and immediate energy release, it may not support sustained energy production, particularly under prolonged heat stress. This metabolic strategy could lead to quick but short-lived energy boosts, potentially exacerbating the negative effects of heat stress on overall cow health and productivity.

Correlation of microbial composition with functional profiles and heat resistance

To further explore how these microbes contribute to the overall resilience of dairy cows under heat stress conditions, the correlation between specific microbial taxa and their functional roles in heat resistance was analyzed. The study identified several key microbial taxa, including *Candidatus Nanosyncoccus alces*, *Clostridiales bacterium 41_21_two_genomes*, *Eubacterium coprostanoligenes*, *Clostridium sp. CAG 678*, *Prevotella sp. CAG 1092*, *Ruminococcus sp. CAG 488*, and *Ruminococcus flavefaciens*. The functional pathways involved include 122 different KEGG Orthology (KO) (Fig. 7).

Candidatus Nanosyncoccus alces is associated with energy metabolism and amino acid biosynthesis, particularly through pathways involving enzymes like PPA (K01507) and arcC (K00926). These functions are vital for maintaining cellular function during heat stress, suggesting a supportive role in the metabolic adaptation necessary for heat resistant. Similarly, *Clostridiales bacterium* contributes to glycolipid metabolism, which is critical for maintaining membrane integrity and ensuring efficient energy utilization under elevated temperatures. *Eubacterium coprostanoligenes* is notable for its role in cholesterol metabolism, involving enzymes like MAN2 C1 (K01191) and E3.1.4.46 (K01126), which is important for lipid homeostasis. Maintaining lipid balance is crucial under heat stress, as it preserves membrane fluidity and function, essential for cellular stability. *Clostridium sp. CAG 678* contributes to carbohydrate metabolism by breaking down complex carbohydrates into simpler sugars through the action of MAN2 C1 (K01191). This process ensures a steady supply of energy, which is

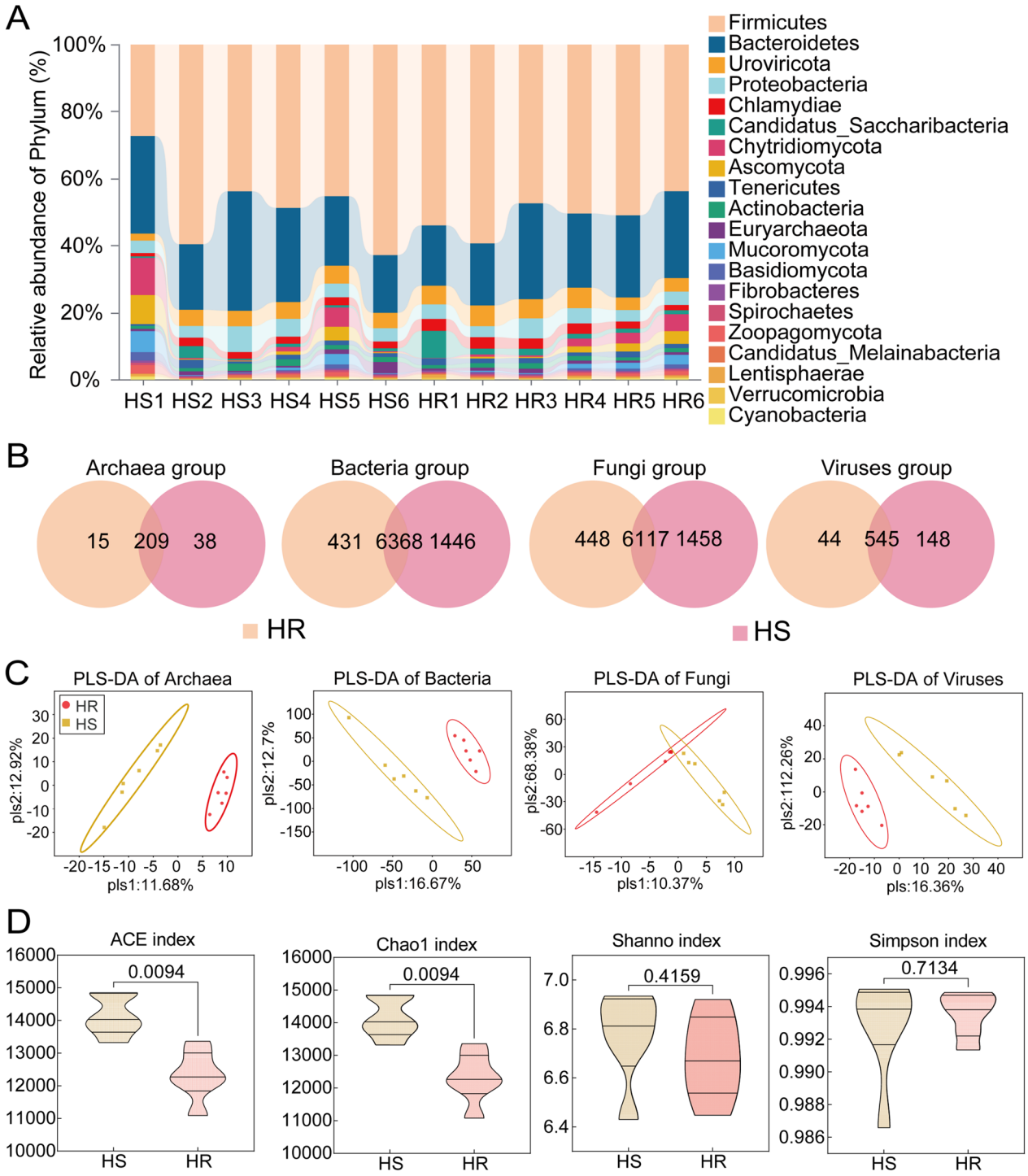

Fig. 3 **A** The top 20 phylum level microbial classes of different samples. **B** Venn diagram showing the composition and relative abundance of rumen microorganisms. **C** PLS-DA analysis based on taxonomic alignment of rumen archaea, bacteria, fungi, and virus. **D** Alpha diversity analysis of rumen microbiota in HR and HS cows. Alpha diversity indices include ACE index, Chao1 index, Shannon index, and Simpson index. Different groups are represented by different colors, with the x-axis showing group names and the y-axis showing diversity index values. The numbers above indicate the *P*-values obtained from the tests, where $P < 0.05$ indicates significant differences between groups, and $P < 0.01$ indicates highly significant differences between groups

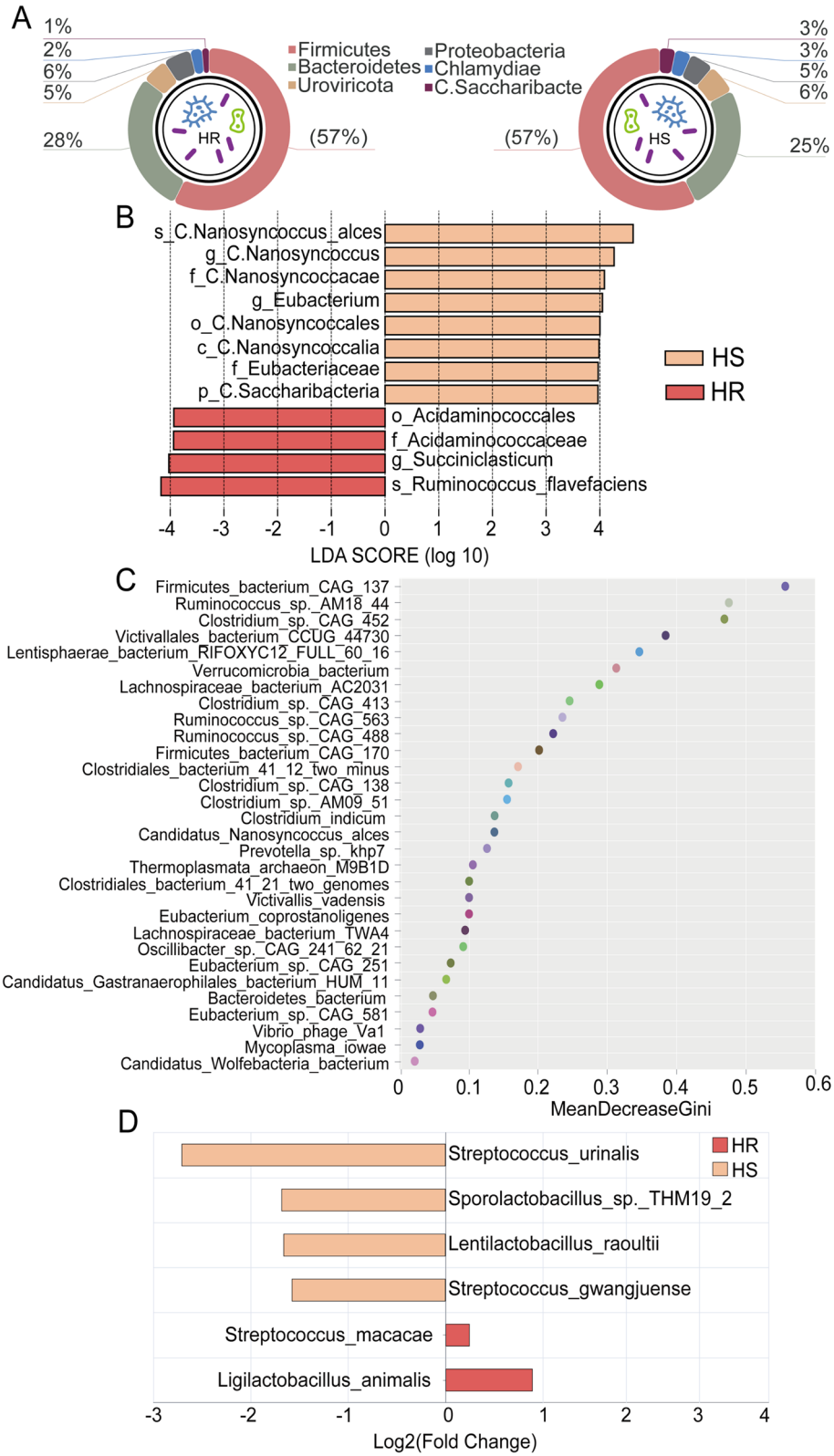

Fig. 4 Differences in rumen microbial composition between cows with different heat resistance. **A** The main components of rumen microbes at the phylum level in HR and HS cows; **B** LEfSe analysis of differential species; **C** Random forest analysis of microbial differences between groups; **D** Changes in the expression of lactate-producing bacteria between HS and HR cows

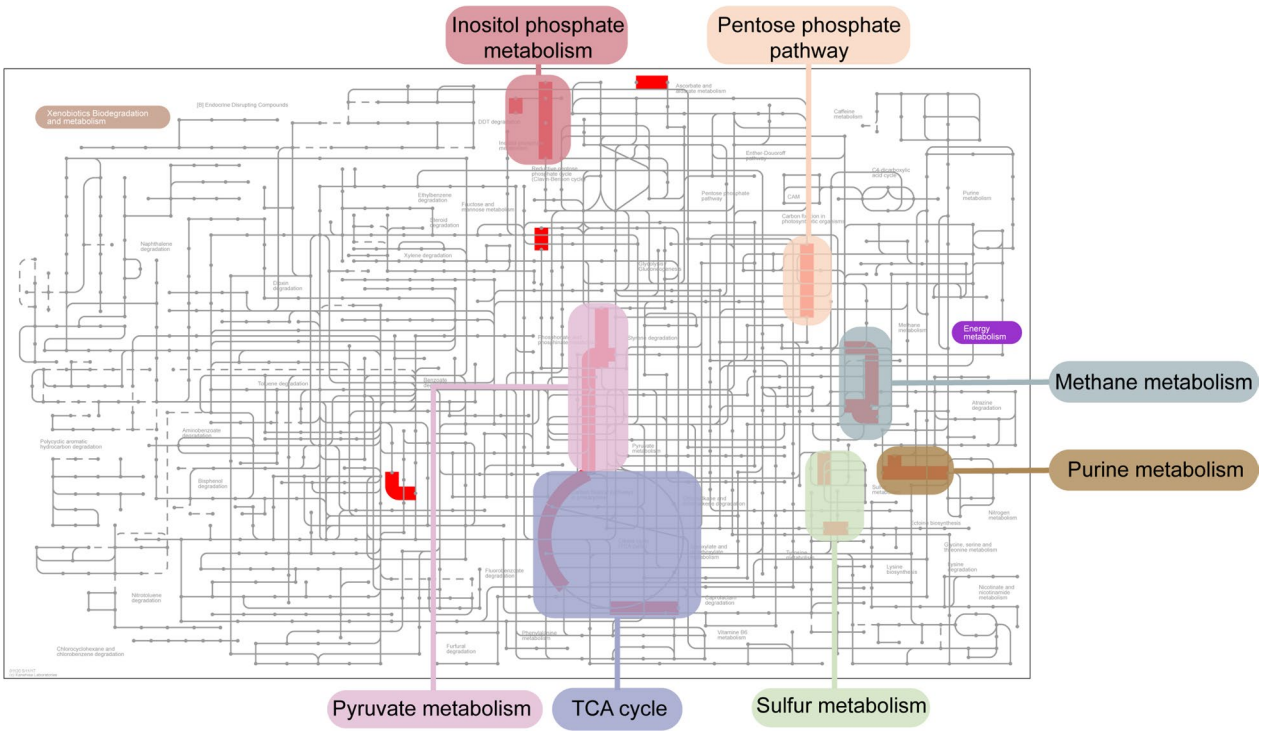

Fig. 5 Visualization of significant differences in KEGG orthologous groups ($P < 0.05$) assigned to the"Microbial metabolism in diverse environments"pathway between HR and HS cows. Red bold lines indicate pathways enriched with differential KOs

crucial for maintaining physiological stability during heat stress, when the energy demands of the host are heightened. Additionally, *Prevotella sp. CAG 1092* is linked to DNA replication processes, particularly through the pol (K02319) gene. This function supports genomic stability and microbial proliferation, ensuring continuous fermentation and energy production under stress conditions.

Ruminococcus sp. CAG 488 and *Ruminococcus flavefaciens*, both key cellulose-degrading bacteria, are essential for the efficient breakdown of fibrous plant material in the rumen. *Ruminococcus sp. CAG 488* is involved in energy metabolism and phospholipid degradation, which are vital for maintaining cell membrane integrity and energy balance. *Ruminococcus flavefaciens*, known for its ability to degrade cellulose, further contributes to the energy metabolism by providing the host with a steady source of fermentable substrates, particularly under heat stress conditions where energy demands are elevated.

Discussion

The results of this study provide valuable insights into the complex interactions between the rumen microbiome and heat resistance in dairy cattle, highlighting the critical role that microbial communities play in mitigating the adverse effects of heat stress. The thermal stress status of dairy cows was accurately assessed using mixed linear models and general linear models, eliminating confounding factors such as days in milk and parity. The application of the entropy-weighted TOPSIS method allowed for the effective differentiation between HR and HS cows, with distinct physiological and biochemical markers confirming the robustness of this classification. Specifically, the reduced salivation index, diminished respiratory rates, and decreased levels of stress biomarkers such as HSP70 and cortisol in HR cows suggest that these animals possess a more stable thermoregulatory and stress response mechanism. The lack of significant differences in RR and RT between the HS and HR groups may be related to the analytical approach of the entropy-weighted TOPSIS model. Single indicators such as RR and RT cannot fully reflect the differences in heat resistance, whereas the entropy-weighted TOPSIS model revealed significant differences in comprehensive scores between the groups by integrating multiple indicators.

The rumen microbiome's composition and functional stability were significantly associated with the heat resistance observed in these cows. HR cows were found to maintain a more cohesive and stable microbial community under heat stress, which is critical for sustaining the efficient breakdown of fibrous feed and maintaining energy balance [45]. The reduced microbial diversity observed in HR cows, coupled with higher functional

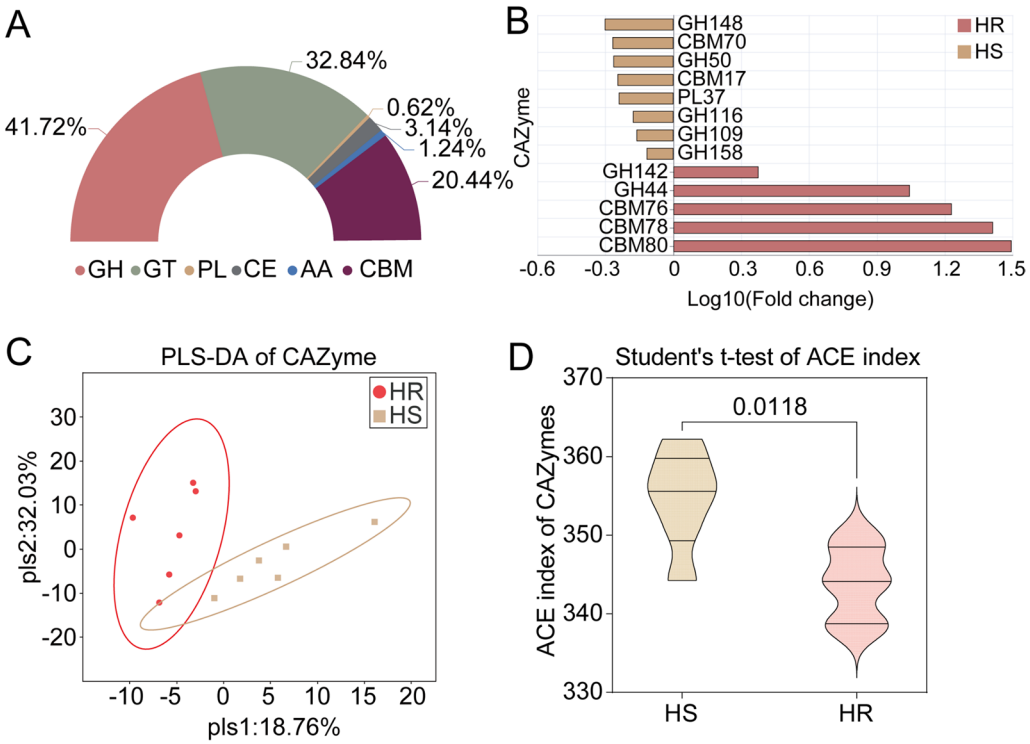

Fig. 6 Changes in the rumen microbial metagenome-encoded carbohydrate-active enzymes (CAZymes) in cows with different heat resistance after exposure to high temperatures. **A** CAZymes encoded by the rumen microbial metagenome; GH = Glycoside Hydrolases; GT = Glycosyl Transferases; PL = Polysaccharide Lyases; CE = Carbohydrate Esterases; AA = Auxiliary Activities; CBM = Carbohydrate-Binding Modules. **B** The top 30 most abundant CAZymes family members by relative abundance. **C** PLS-DA analysis of CAZymes families. **D** ACE diversity analysis of CAZymes families

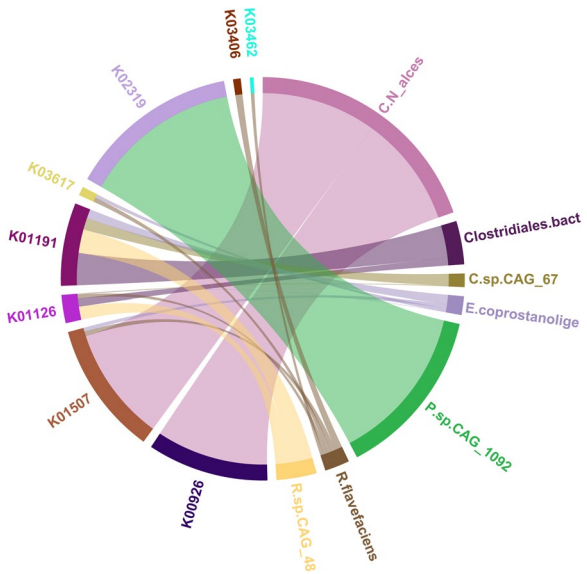

Fig. 7 Key microbial and functional correlation analysis based on different heat resistance levels. The rumen microbes and KO functions regulated by heat resistance show significant differences ($P < 0.05$). The width of the connecting lines indicates the extent to which a particular bacterium contributes to a specific function

stability (as indicated by lower Shannon and Simpson indices), suggests that a less diverse but more specialized microbiome might confer advantages in adapting to heat stress. This contrasts with HS cows, where greater microbial diversity was observed but with less functional coherence, potentially indicating a disrupted and less efficient microbial ecosystem under stress.

The dominance of *Firmicutes* and *Bacteroidetes*, which are integral to rumen fermentation processes, aligns with existing literature that underscores the importance of these phyla in maintaining digestive efficiency and overall metabolic health in ruminants [46, 47]. However, the presence of specific taxa such as *Ruminococcus flavefaciens* and *Succiniclasticum* in HR cows is particularly noteworthy. These microbes are key players in the degradation of complex carbohydrates and the production of SCFAs, essential for maintaining energy homeostasis and intestinal health, especially under the duress of heat stress [48, 49]. The functional importance of these microbes suggests that their presence might be a marker of enhanced heat resistance, as their metabolic activities support the energy needs of the host during periods of environmental stress [50].

In contrast, HS cows were characterized by a microbiome that favored lactate-producing bacteria such as *Streptococcus* and *Lactobacillus* [51, 52]. These genera are associated with lactate production, which can lead to an acidogenic rumen environment, predisposing cows to SARA [53]. This condition not only disrupts the rumen pH balance but also exacerbates the physiological strain on the cows, compounding the negative effects of heat stress. The shift towards a more acidogenic microbiome in HS cows might, therefore, contribute to their reduced heat resistance, as the rumen environment becomes less conducive to efficient digestion and energy extraction.

The functional pathway analysis further supports these findings. The enrichment of the PPP in HR cows highlights a critical metabolic adaptation that enhances their ability to manage oxidative stress, a common consequence of heat stress [54, 55]. By generating NADPH, the PPP helps protect cells from oxidative damage, ensuring that cellular functions are maintained even under challenging conditions [56]. This metabolic resilience likely contributes to the overall robustness of HR cows, allowing them to sustain productivity and health during periods of elevated temperatures. On the other hand, the greater reliance on energy-intensive pathways such as the TCA cycle and pyruvate metabolism in HS cows suggests a less efficient metabolic strategy under heat stress [57]. While these pathways are crucial for ATP production, their upregulation in HS cows might reflect an increased metabolic burden that, in turn, heightens oxidative stress and cellular damage. This metabolic inefficiency could lead to the observed decline in health and productivity in HS cows under prolonged heat stress conditions.

The differential expression of CAZymes between HR and HS cows further illustrates the functional divergence in their rumen microbiomes. The upregulation of glycoside hydrolases and carbohydrate-binding modules (CBMs), particularly CBM80, in HR cows indicates a microbial community well-adapted to the efficient breakdown of fibrous feeds. This capability is especially important during heat stress, when maintaining energy balance is critical to preventing metabolic disorders. In contrast, the CAZyme profile in HS cows, which favors the breakdown of simpler sugars, suggests a microbial community more adept at rapid but unsustainable energy production. This short-term energy strategy may not support the long-term metabolic needs of the host under sustained heat stress, potentially exacerbating the negative impacts on health and productivity.

The implications of these findings are significant for the development of strategies to enhance heat resistance in dairy cattle. The identification of specific microbial taxa and functional pathways associated with heat resistance offers new avenues for selective breeding, dietary interventions, and microbiome-targeted therapies aimed at enhancing the resilience of dairy cows to heat stress. Future research should focus on validating these microbial markers in larger and more diverse populations, as well as exploring the potential for manipulating the rumen microbiome to improve heat resistance.

Conclusion

In conclusion, this study demonstrates that the rumen microbiome plays a pivotal role in mediating heat resistance in dairy cattle. The distinct microbial compositions and functional pathways observed in HR and HS cows provide a foundation for developing microbiome-based strategies to mitigate the effects of heat stress. HR cows exhibited a functionally stable microbiome enriched with fiber-degrading bacteria, which supports efficient energy production and oxidative stress management under thermal stress. Additionally, while our findings suggest associations between specific microbial profiles and heat resistance, causality cannot be firmly established without further experimental validation. Future research should aim to isolate the direct effects of heat stress to explore microbial markers associated with heat resistance and investigate microbiome manipulation to enhance cattle resilience to rising global temperatures.

Abbreviations

HR	Heat-resistant
HS	Heat-sensitive
NTN	Non-thermoneutral
TN	Thermoneutral
RT	Rectal temperature
RR	Respiratory rate
SI	Salivation index
K^+	Potassium ion
HSP70	Heat shock protein 70
THI	Temperature-humidity index
PPP	Pentose phosphate pathway

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Author contributions

W.ZW crafted the initial draft of the manuscript,M.Z and C.P developed the comprehensive outline for the text, Z.L and J.HR contributed to the consultation of relevant literature, Y.ZP and M.YJ conducted a thorough review, respectively. L.MX engaged in a critical revision of the full review to ensure a complete and extensive knowledge base. All authors participated in reading and revising multiple versions of the draft, ultimately approving the submission of the final manuscript.

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Availability of data and materials

All raw sequencing data that support the findings of this study have been deposited in the Sequence Read Archive (SRA) under the accession number

PRJNA1172760, https://dataview.ncbi.nlm.nih.gov/object/PRJNA1172760reviewer= t94qq9780 d8k3oofhb730bbp7 t.

Declarations

Ethics approval and consent to participate

All procedures in this experiment were conducted according to the Animal Protection Law based on the Guide for the Care and Use of Laboratory Animals approved by the Ethics Committee of Yangzhou University.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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