WITHDRAWN: Novel Insights Into Heat Tolerance Using Microbiome and Metabolomics Analyses in Dairy Cows Rumen Fluid

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Abstract

Background: Heat stress is a key issue of growing concern for livestock industry worldwide due to its negative effects not only on milk production, fertility, health, welfare, and economic returns of dairy cows, but also on the microbial communities in the rumen. However, the underlying relationship between rumen microbiome and its associated metabolism with heat tolerance in cow have not been extensively described yet. Therefore, the main objective of this study was to investigate differential heat resistance in Holstein cows using rumen microbiome and metabolome analyses.

Methods: We performed both principal component analysis and membership function analysis to select 7 heat-tolerant (HT) and 7 heat-sensitive (HS) cows. The ruminal fluid samples of two groups were collected at two hours post feeding on 7th day of heat stress period, for analyses including rumen fermentation parameters, rumen microbiome and nontargeted metabolomics.

Results: Under heat stress conditions, the HT cows had a significantly higher propionic acid content than the HS cows; whereas measures of the respiratory rate (RR), rectal temperature (RT), acetic,

butyric acid and acetic acid to propionic acid ratio (A:P) in the HT cows were lower compared with the HS cows. Omics sequencing revealed that the relative abundance of *Rikenellaceae_RC9_gut_group*, *Succiniclasticum*, *Ruminococcaceae_NK4A214_group and Christensenellaceae_R-7_group* were significantly higher in the HT than HS cows; whereas *Prevotella_1*, *Ruminococcaceae_UCG-014*, and *Shuttleworthia* were significantly higher in the HS cows compared to HT cows. Substances mainly involved in carbohydrate metabolism, including glycerol, mannitol, and maltose, showed significantly higher content in the HT cows compared to that in the HS cows. Simultaneously, RR was significantly correlated with both differential microorganisms and distinct metabolites, suggesting three metabolites could be potential biomarkers for determining heat resistance that require further research.

Conclusion: Overall, distinct changes in the rumen microbiome and metabolomics in the HT cows may be associated with better adaptability to heat stress. These findings suggest their use as diagnostic tools of heat tolerance in dairy cattle breeding schemes.

Background

With the continual increment in the global warming, heat stress has always been an emerging and rising problem for several livestock production systems in the world, including the dairy industry in particular regions of China. Negative effects of heat stress on animal performance are widely recognized. Excessive ambient temperature and humidity have been associated with poor milk yield and quality, inferior reproductive performance, impaired immune function, increased mortality, and reduced feed intake of dairy cows [1–4]. Heat stress is even more a challenge for high yielding dairy cows, causing a huge economic loss each year [5]. Such high milk producing cows have a poor heat dissipation capacity with greater metabolic heat production that makes them particularly sensitive to changes in ambient temperature [6]. Mitigation of heat stress on animal performance can be achieved through different

procedures, including physical protection, feeding regime, and genetic improvement [7]. Supported by the increased animal management costs and innovative molecular genetics technologies, breeding heat-tolerant (HT) cows seems to be the best solution and cost-benefit option to alleviate heat stress so that improve animal productivity and welfare [8]. Respiratory rate (RR) is a reliable indicator of heat tolerance, together with rectal temperature (RT) [9]. In addition, the reduction of milk or blood biochemical indicators such as heat shock proteins (*HSPs*), cortisol hormone and erythrocyte potassium ion content are the most frequently parameters used as reference indicators for screening heat tolerance [10, 11].

As a bioreactor, rumen microorganisms are closely related to cow performance and body health and are susceptible to heat stress [12]. Data point out that 70% of the daily energy demand of dairy cows depends on fermentation by-products (e.g., short-chain fatty acids) produced by rumen microorganisms, and 50% of the daily protein demand is derived from the microorganisms themselves [13]. Microbiome sequencing was used to determine changes in the rumen bacterial taxa of Jersey and Holstein cows to heat stress [14]. Several experiments have already been conducted to measure some metabolites of heat stress in dairy goats [15], pigs [16] and dairy cows [17]. In addition, Tian [18] reported 10 biomarkers in milk associated with heat stress-induced metabolomic alterations in blood. In parallel, metabolites in milk and plasma help detect that Xuanhan Yellow cattle breed, whose glycolysis process has been strengthened, was more adaptable to the thermal environment than other breeds[19].

To date, none of the articles comparing the heat resistance of the same breed from the microbiome and metabolites level in rumen. In this experiment, a mathematical method was implemented to measure multiple heat tolerance-related indicators to differentiate between HS cows and HT cows, and uses rumen gas chromatography metabolomics (GC-MS) and rumen 16S rDNA multi-omics methods which further investigate the potential contribution of rumen microbial diversity and low-molecular-weight metabolite systems to the observed difference in heat tolerance of dairy cows, thereby affecting milk production. This would provide an efficient basis for genomic selection and breeding of high-yielding HT dairy cattle.

Methods

Animal Management

The trial was conducted in the last week of May and first week of July 2020 at Xincai Ruiya Animal Husbandry Co., Ltd (Zhu Madian, China), all data were collected in the experimental period. A total of 54 third-parity Holstein cows were chosen for the experiment. Holstein cows averaged a body weight of 627 ± 47.4 kg, an age of 52 ± 0.2 mon, and a milk yield of 46 ± 0.75 kg/d (49 ± 1.1 Day in milk (DIM)) in May and 42 ± 0.74 kg/d (86 ± 1.1 DIM) in July. Experimental animals were housed in a shaded open barn under natural lighting conditions. During the 7-day trial, the diets of cows were formulated to meet or exceed the energy requirements and reared on TMR twice a day. Fresh drinking water was ensured for the cows and the diet was offered to ensure 5% refusals, there were no orts from any of the cows. The TMR composition (DM basis) was 47.1% corn silage, 22.5% concentrated, 7.2% steam-flaking corn, 1.0%

soybean meal, 1.0% pelleted beet pulp, 12.3% brewer's grain, 6.2% alfalfa hay, 2.1% oat hay, 0.4% sodium bicarbonate, and 0.2% Yikang XP. (Hefeng Feed Co. Ltd.).

Measurement of Relevant Indicators of Heat Resistance

In order to examine the impact of heat stress on the microbial community within the rumen, two different time points were specified based on temperature-humidity index (THI). The parameters of dairy cows including milk production and physiological function were collected at both non-heat stress (May) and heat stress (July) period, the change of milk yield in July compared with May was also calculated for the HT and HS cows, respectively. The temperature and humidity of cowshed were monitored every hour during the experiments. The following formula was applied to calculate THI [20]:

$$THI = (1.8 \times AT + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times AT - 26)], \quad (1)$$

where AT is the average ambient temperature (℃); and RH is the average relative humidity (%). To determine the degree of heat stress, RR and RT were periodically measured at 12:00 am and 3:00 pm during the whole experimental period, as previously described by Scharf [21]). Meanwhile, a single blood sample (10 mL) from each cow was taken at 2:30 p.m. within a 7-day window for plasma separation. Then, blood samples were centrifugated at 3500 r/min for 10 min and stored at -20 ℃ until performing the analyses of plasma cortisol, heat shock protein 70 (*HSP*70), heat shock protein 90 (*HSP*90), and potassium content in erythrocytes using commercial kits from Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China. All assays had intra- and inter-assay coefficients of variation of less than 10 and 15%, respectively.

Comprehensive Evaluation on Heat Tolerance Related Traits

Mathematical methods were used to evaluate the heat tolerance of 54 cows. There were seven heat tolerance traits including milk yield variation, RR, RT and blood parameters. The retained principal components were calculated by the membership function after principal component analysis based on the principle of a cumulative variance contribution rate ≥ 70. The calculation formula is as follows:

$$R(Zi) = (Zi - Zmin)/(Zmax - Zmin) \quad i = 1,2,\ldots,n \quad (2)$$

where *Zmax* and *Zmin* are the maximum and minimum values of the principal component, respectively; *Z* is the principal component.

$$Wi = Pi/\sum_{i=1}^{n} Pi \quad i = 1,2,\ldots,n \quad (3)$$

where Pi is the principal component's contribution rate; Wi is the weight of this principal component in the first m principal components used to evaluate heat tolerance in dairy cows.

$$RW = \sum_{i=1}^{n} [R(Zi)] \times Wi \quad i = 1,2,\ldots,n \quad (4)$$

where RW is the weighted membership value calculated by the principal component, which is used to rank the heat tolerance of the experimental animals.

Collection of Rumen Fluid and Laboratory Measurements

The rumen fluid was collected once per cow after two hours of morning feeding, according to the method described by Liang [22]. Rumen fluid samples from 7 HT and 7 HS cows were extracted from the rubber end of the rumen tube connected with a syringe posterior to discarding the first two times of extraction for minimizing saliva contamination. After measuring the pH value, the collected 20 mL of the rumen fluid was dispensed into two 10 mL cryogenic tubes (one copy for rumen fermentation parameters analysis and another one for omics sequencing). Immediately, rumen fluid samples were frozen at -80 ℃ until subsequent analyses. Volatile fatty acids (VFA) were determined by ion chromatography using a Dionex ICS-3000 ion chromatograph following the procedure mentioned by Li [23] and Seankamsorn [24]. Ammonium nitrogen (NH_3-N) in rumen fluid was determined by phenol-sodium hypochlorite colorimetric method [25] (TU-1810, Beijing Puxi General Instruments Co., Ltd.). Then, a total of 14 rumen fluid samples were sent to Guangzhou Omicshare Biotechnology Co. Ltd., for sequencing.

The results of microbial sequencing were as follows: After genomic DNA was extracted from the samples, the V3-V4 regions of the 16S rDNA was amplified by PCR with specific primers 341F (5’-CCTACGGGNGGCWGCAG-3’) and 806R (5 '-GGACTACHVGGGTWTCTAAT-3') with barcode. The purified amplification products were mixed in equal amounts, ligated with sequencing adapters, and the sequencing library was constructed and sequenced on IlluminaPE250 (Guangzhou Gene Denovo Biotechnology Co., Ltd.). To ensure obtaining high accuracy of further analyses, we filtered the low-quality reads of sequences according to the following rules: reads containing more than 10% unknown nucleotides, and reads containing less than 80% of bases with quality more than 20 were removed. Followed by assembly and re-filtering to obtain valid data for operational taxonomic unit (OUT) clustering using UPARSE (version 7.1). Analysis of differences in two grouped species (filtered for species with a sum of abundances below 0.1% in all samples on phylum and species level) was performed with Welch's T-test.

Agilent 7890 gas chromatograph system coupled with a Pegasus HT time-of-flight mass spectrometer (LECO, St,Joseph, MI) were used to conduct GC-MS analyses of samples. A DB – 5 MS capillary column was 30 m × 250 μm × 0.25 μm (J & W Scientific, Folsom, CA, USA), the injection temperature was 280 ℃ and the gas flow rate through the column was 20 mL/min. The QC samples were inserted into the samples to monitor system stability and data quality, the GC-MS data analysis was conducted by integrating each resolved chromatogram peak. Significantly differential metabolites were screened using variable importance in projection (VIP) scores (VIP > 1) from the Orthogonal partial least squares discriminant analysis (OPLS-DA) and *P*-values ($P < 0.05$). The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to find the metabolic pathways and enrichment analysis.

Statistical Analysis

Pearson correlation analyses were measured with the vegan package of R software (Version 3.2) to analyze the association of heat tolerance indexes, rumen microorganisms and nutrient metabolites. The data on milk yield variation, physiological indicators, blood parameters and rumen fermentation were

analyzed with Student's t test by SPSS. The results of these analyses are expressed as the means and standard errors of the means (SEM). $P < 0.01$ was considered highly significant and a probability of $P < 0.05$ was the minimum level of significance.

Results

Selection of HT Cows and HS Cows

The overall average daily THI was 67.73 ± 0.95 and 84.16 ± 0.57 during the normal (May) and heat stress (July) conditions, respectively (Fig. 1). The highly significant ($P < 0.01$) difference between May and July in terms of temperature, humidity, and THI, indicates that cows were continuously exposed to heat stress during the July trial. As shown in Table S1, the accumulated variance contribution ratio of principal components 1–4 was approximately 0.71, which can represent most of the seven indicators' information, thereby establishing an evaluation system to comprehensively evaluate the heat tolerance in dairy cows. In light of the magnitude of the weighted membership function value (RW) for sorting the heat tolerance of the 54 cows under study, the top 7 cows with higher RW values were considered as HS cows, and the bottom 7 cows were classified as HT cows (Table S2).

Heat Stress Response Differences between HT Cows and HS Cows

The parameters reflecting animal susceptibility to heat stress are demonstrated in Table 1. Obviously, the HT and HS cows showed differential stress responses in heat stress period during July. The physical parameters, including RR ($P < 0.05$) and RT ($P < 0.01$), showed lower values in the HT cows than HS cows, indicating that the grouping method is accurately representative. It also reveals a sensitivity of HS cows to severe heat stress conditions. The selected HT cows had highly significantly ($P < 0.01$) lower acetic acid to propionic acid ratio (A:P) than HS cows. Compared with cows in HS group, the HT cows had a significantly ($P < 0.05$) higher level of propionic acid and a significantly ($P < 0.05$) lower levels of acetic acid and butyric acid. However, the two studied groups did not show significant difference in milk yield variation as a consequence of the heat stress conditions. Whether the microorganisms and their metabolic alterations in rumen are associated with heat tolerance in dairy cows needs further investigation. This encouraged us to screen the microbial community in the rumen during heat stress.

Rumen bacteria diversity in HT cows and HS cows

The results of the alpha diversity index analysis are presented in Fig. 2, both Ace and Sobs indices were significantly ($P < 0.05$) greater in the HT cows than HS cows. On the other hand, the Chao 1 index had a significantly ($P < 0.01$) higher value in the HS cows compared to HT cows. These results indicated that the species richness of the HT cows was better than that of the HS cows, whereas the species evenness of the two groups did not differ significantly. PCoA based on the Bray-Curtis distance matrix at the genus

level showed that both HS and HT cows were significantly aggregated, indicating that the rumen microflora of the HT and HS cows are different (Figure S1).

The composition and relative abundance of rumen microbes at phylum and genus levels are shown in Fig. 3. The taxonomical analysis of the ruminal bacteria revealed that the dominant bacterial phyla (average relative abundance ≥ 1) involved *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. The differential abundance comparison analysis at the phylum level by Welch's t test did not show significant differences in the top dominant bacteria, except for *Tenericutes*, between the HT and HS groups of cows. Analysis of the top ten microorganisms at the genus level showed that seven genera differed significantly between the two groups. There were four bacteria significantly ($P < 0.01$) higher in the HT cows including *Rikenellaceae_RC9_gut_group*, *Succiniclasticum*, *Ruminococcaceae_NK4A214_group and Christensenellaceae_R-7_group*; whereas *Prevotella_1*, *Ruminococcaceae_UCG-014* and *Shuttleworthia* were significantly ($P < 0.01$) higher in the HS cows.

Influence of Environmental Factors on Microbial Community Structure

The relationship between rumen fermentation characteristics, sampled animals, and microbial communities through redundancy analysis (RDA) is exhibited in Fig. 4. Sample clustering in Fig. 4 is consistent with grouping, indicating that the species and environmental effects on the sample distribution is in parallel with the grouping effect. The longest arrow was corresponding to propionic acid, indicating that propionic acid has the greatest impact on the microbial species distribution in the rumen. The Pearson correlation analysis at the genus level (Figure S2) demonstrated that the relative abundances of *Rikenellaceae_RC9_gut_group*, *Succiniclasticum*, *Ruminococcaceae_NK4A214_group* and *Christensenellaceae_R-7_group* were positively correlated with propionic acid ($P < 0.05$), and negatively correlated with A:P ($P < 0.05$). In contrast, *Ruminococcaceae_UCG-014* was negatively associated with propionic acid ($P < 0.05$).

Differential Metabolites Identification and the Analysis of the Metabolic Pathway

Samples of the two studied groups were clearly separated clearly according to OPLS-DA analysis, and the principal component analysis (PCA) provided a more extensive explanation (Figure S3). There was a difference in the expression of metabolites between HS and HT groups. Rumen metabolomics analysis demonstrated that there were 56 metabolites identified among the two groups. These metabolites are mainly carbohydrates, nucleotide, amino acid, and lipid. All 56 metabolites were classified into 22 KEGG second-grade pathways. The 20 differential metabolites obtained by t-test were significantly higher in the HT cows than HS cows ($P < 0.05$). To better evaluate how multiple metabolites differed between the HS and HT cows, the selected differential metabolism was tested by t-test between the two groups, and only metabolites with $P < 0.05$ and VIP > 1 were considered as potential biomarkers (Table 2). The result

showed that rumen fluid from the HT cows contained higher levels of maltose, glycerol, and mannitol compared with the HS cows. These three biomarkers are mainly involved in carbohydrate metabolism, membrane transport, and lipid metabolism.

The Correlation Analysis between Heat Tolerance Indexes, the Rumen Microbiome and Metabolites

Pearson correlation analysis was carried out to assess the relationships between heat tolerance indexes, rumen fermentation parameters and metabolites. RR was negatively correlated with differential metabolites. Conversely, propionic acid was positively correlated with differential metabolites. In order to further analyze which species and associated metabolites produce group differences from the huge microbial and metabolite populations, association analysis between microbiome and metabolites was performed using Pearson correlation coefficient. We found that three differential metabolites were positively correlated with *Rikenellaceae_RC9_gut_group*, *Succiniclasticum*, *Ruminococcaceae_NK4A214_group*, *and Christensenellaceae_R-7_group* at genus level; whereas only maltose had negatively correlation with *Prevotella_1* and *Ruminococcaceae_UCG-014* at genus level.

Discussion

Heat stress as a potential threat to dairy cows during the summer months is globally well recognized. Environmental temperature and humidity are both critical factors for evaluating the comfort of dairy cows, and THI 68 is widely used to determine the margins of the thermoneutral zone for dairy cows [26]. Compared with May, July had significantly higher temperature and humidity. Moreover, THI was above 80, indicating that the cow herds to be tested were subjected to a state of continuous heat stress. In the current study, other heat tolerance related indicators were all major contributors among the first four principal components retained, except for potassium ion content. *HSPs* are a group of proteins rapidly synthesized by the animal body under heat stress conditions, and play a major role in biological heat tolerance. For instance, when environmental changes generate stress, *HSP*70 can play a major role in modulating inflammation through enhancing the tolerance of cells to the next harmful injury [27]. Cortisol is related to metabolism, and shows continuous changes, so it is mostly used as a reference indicator of heat stress [26]. The alteration in milk yield of dairy cows, as the most practical indicator for measuring heat resistance, is affected by many aspects such as management practice and lactation number of dairy cows, so it has critical limitations [28]. Apparently, HS animals are known to have increased energy expenditure for heat loss mechanisms via panting and sweating, as shown by the elevation of RR and RT [29]. In our trial, only physiological measures related to heat tolerance were significantly different between the two groups. Estimates of RR and RT in the HT cows were lower than the HS cows, indicating that the HT cows had superior thermoregulatory capacity and less response to heat challenge, which confirm that our adopted grouping strategy was considerably accurate.

Heat stress affects the microbial environment in the rumen, resulting in an increase in pathogenic microorganisms, slowing down of adaptation pathways to the environment, and disorders of immune

response pathways and metabolic pathways [30]. The concentration of rumen VFA depends on microbial fermentation efficiency, and host absorption rate [31]. In the current study, there were significant differences in acetic, propionic and butyric acid levels between the two groups, even A:P, indicating that the rumen fermentation pattern was distinct between both HT and HS cows. Rumen as an important nutrient digestion and absorption site in ruminant animals, the resulting products of fermentation performed by the rumen microorganisms are VFAs (mainly acetic, propionic and butyric acids), which directly associated with epithelial absorption by the animal [32]. Among them, acetic acid is an important substrate that has a greater impact on milk fat synthesis; whereas propionic acid is involved in lactose synthesis in milk through the liver, as a major substrate for gluconeogenesis [33]. Moreover, since propionate is the predominant glucose precursor in ruminants, elevations in glucose stimulate milk protein synthesis [34]. We speculate that the differential basic metabolism of VFAs in the HT and HS cows would cause variation in milk quality. Overall, such changes in VFA concentration demonstrate that difference in heat resistance may affect rumen fermentation by shifting the structure and function of the microbial communities within the rumen.

In order to figure out the possible mechanism of rumen parameters whether impacts the heat resistance, we measured the microbiota community via 16S rDNA sequencing technology. For the differential abundance comparison analysis at the phylum level, the ratio of *Firmicutes* to *Bacteroidetes* was found to be positively correlated with milk fat in dairy cows, as reported by Jami et al[35]. *Firmicutes* showed higher abundance with *Bacteroidetes* in both HS cows and HT cows, which is in agreement with most previous studies, but the difference between the two groups was not significant. Meanwhile, alpha diversity results obtained in this study indicated no significant difference in the species richness of the two groups. It could be, therefore, speculated that although the change of rumen bacteria in the HT and HS cows is closely related to the change of warm environment, the different adaptability of the two groups of cows to ambient environmental changes may be largely caused by the alteration in some rumen microorganisms, rather than the change in the overall bacterial diversity in the rumen.

We observed some genus of *Firmicutes, including Ruminococcaceae_UCG-014*, and *Prevotella_1* has an enrich relative abundance in the HS cows compared with the HT cows, whereas *Ruminococcaceae_NK4A214_group*, and *Christensenellaceae_R-7_group* have higher abundance in the HT group than the HS cows. These genus were related to eating habits [36]. In particular, *Ruminococcaceae* taxa plays an important role in cellulose and hemicellulose degradation as well as rumen biohydrogenation pathways, where the enhancement of hemicellulose degradation can improve feed utilization and ultimately improve animal performance [37]. Therefore, both HT and HS cows under heat stress conditions may have significant differences in feed intake and uptake of nutrients such as fat and protein. Such specific mechanisms of action during heat stress needs further investigation. In Chinese sheep, Mi [38] found that a negative correlation between *Succiniclasticum* and methane emissions. Not only *Succiniclasticum*, but also *Prevotella_1* and *Rikenellaceae_RC9_gut_group* were able to reduce the methanogenesis rate. Importantly, *Succiniclasticum* can produce propionic acid by succinic acid deacidification compete with methanogens for hydrogen and contribute to the attenuation of methanogenesis [39]. *Rikenellaceae_RC9_gut_group* was involved in the VFA production and the

scavenging of H_2 [40]. The HT cows may also inhibit rumen fermentation and reduce methane emission in the rumen environment by inhibiting protein and fiber degrading bacteria represented by *Prevotella_1* [41]. In addition, *Rikenellaceae_RC9_gut_group*, which belong to the Bacteroidetes phylum, is closely related to the metabolism of thyroxine [42]. *Rikenellaceae_RC9_gut_group* was negatively correlated with RR and RT measured in the present study; whereas it correlated positively with propionic acid that had the greatest effect on the bacterial species distribution in the HT cows as shown by the RDA analysis. This is consistent with the fact that propionic acid, which is a substrate for gluconeogenesis, activates gluconeogenic gene expression to maintain energy homeostasis [43]. It is speculated that *Rikenellaceae_RC9_gut_group* plays an essential role in heat production and body temperature regulation in the HT cows, and promotes the recovery of the metabolic rate in heat stress environment. The results mentioned above indicate that HT cows can reduce methane emission and decrease heat production. Briefly, these differential genera play a crucial role in the basic metabolic process in the HT cows.

Metabolites reflect alterations in the metabolism of dairy cows, which can enable a comprehensive understanding of an organism's physiological and biochemical status. High-yielding dairy cows under the heat stress conditions noticeably consume large amounts of glucose for fat mobilization and are mostly in a negative energy balance state. According to previous reports, cows can alleviate the negative energy balance through the activity of fatty acid oxidation, and glycerol catabolism pathways [44]. In the current study, we found that the contents of maltose, glycerol, and mannitol in the HT cows increased compared with the HS cows, where these metabolites were mainly involved in carbohydrate metabolism. It is worth noting that glycerol, as an important precursor of gluconeogenesis, provides energy to cells through glycolysis in the liver [45]. Simultaneously, glycerin and long-chain fatty acids form triglycerides, which are important component involved in milk fat synthesis [46]. Agreed with that reported by Wheelock [47], we speculate that the HT cows may minimize the adipose tissue triglyceride mobilization allowing for a stronger ability to alleviate the negative energy balance they suffer from. According to Robergs and Griffin [48], glycerol intake contributes to improved thermoregulation and heat tolerance ability when humans are exposed to hot environments. Similarly, Kim [14] has reported that Holstein cows had higher RT and RR than Jersey cows under the heat stress condition of THI of 87.5, providing that the last breed is less susceptible to heat stress, and has more carbohydrate-related metabolic pathway genes. Consistent with previous reports, our data demonstrate that differential metabolites (glycerol, mannitol, and maltose) were negatively correlated with RR, since lower RR values indicated to the thermoregulatory ability of the HT cows, which were more advantageous than the HS cows in terms of body heat production and heat dissipation, and showed better adaptability to harsh environments. Furthermore, maltose and mannitol were positively correlated with *Rikenellaceae_RC9_gut_group.* It is worth noting that *Rikenellaceae_RC9_gut_group* can degrade structural carbohydrates and starch in the rumen of dairy cows and plays a key role in carbohydrate metabolism [49]. This would imply that a shift of the metabolic pathways of microbes may be related to carbohydrate degradation. Under the same dietary level and feeding conditions, HT cows may convert microorganisms in the rumen into VFAs through carbohydrate metabolism [50], in order to obtain the carbon skeleton of gluconeogenesis and improve their own heat resistance and by providing metabolic energy required for rumen microbiota.

Conclusion

The data of the present study referring to physiological parameters show that the HT cows had better response to heat stress as indicated by lower RR and RT, which were associated with alterations in the microbial composition and differential metabolites. The structures of rumen bacterial communities, including *Rikenellaceae_RC9_gut_group*, *Succiniclasticum*, and *Ruminococcaceae_NK4A214_group* in the HT cows were significantly higher than the HS cows, indicating vital changes in the rumen floras of the HT cows to adapted to heat stress environment. A total of three metabolites were considerably altered in the HT cows compared with the HS cows. Maltose, glycerol, and mannitol were mainly involved in carbohydrate metabolism, and therefore could be potential diagnostic biomarkers for thermal adaptability. Interactions between physical parameters, rumen microorganisms, and metabolites may partially explain the resistance of the HT cows to thermal environments.

Abbreviations

HT: Heat-tolerant; RR: Respiratory rate; RT: Rectal temperature; HSPs: Heat shock proteins; GC-MS: Gas chromatography metabolomics; DMI: Day in milk; THI: Temperature-humidity index; HSP70: Heat shock protein 70; HSP90: Heat shock protein 90; VFA: Volatile fatty acids; NH3-N: Ammonium nitrogen; OUT: Operational taxonomic unit; VIP: Variable importance in projection; OPLS-DA: Orthogonal partial least squares discriminant analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; RW: The weighted membership function value; A:P: Acetic acid to propionic acid ratio; RDA: Redundancy analysis; PCA: Principal component analysis

Declarations

Authors' contributions

LZL, FLP and ZZ helped with the sample collection and measurement. ZCW and LZL created and carried out the analysis, interpreted the data. ZCW wrote the manuscript. SHL, HER and FL conceived and designed the experiments. ZWT and SHL reviewed the paper. TYG, LGY, TM and TF contributed reagents, materials, and analysis tools. HER critically revised the draft and significantly contributed to reviewing the final article. SHL and TYG managed the project and was responsible for funding acquisition and supervision. All authors read and approved the final paper.

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Availability of data and materials

All the analyzed datasets in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Animal care and experimental procedures were conducted according to the guidelines of the Animal Care and Use Committee of Henan Agricultural University, Henan Agricultural University, Zheng Zhou, China (Approval number: HENAU-2020-015).

Consent for publication

All the authors read and agree to the content of this paper and its publication.

Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1 Heat tolerance indexes and rumen fermentation parameters of heat-tolerant cows (HT cows; n=7) and heat-sensitive cows (HS cows; n=7)

Index[1]	HT cows	HS cows	SEM	*P*-value
Production performance				
Milk yield variation (kg)	-7.07	-3.02	2.033	0.070
Physiological indicators				
Respiratory rate (bpm)	69.24	99.17	3.659	0.000
Rectal temperature (℃)	38.96	39.41	0.154	0.018
Biochemical indexes				
Potassium content in erythrocytes(ng/mL)	2.81	3.01	0.115	0.122
Plasma cortisol (ng/mL)	142.19	178.45	18.929	0.091
*HSP*70 (ng/mL)	427.93	433.31	52.005	0.919
*HSP*90 (ng/mL)	286.1	295.51	26.228	0.726
Rumen fermentation				
pH	6.65	6.52	0.134	0.345
Acetic acid(mmol/L)	59.64	69.07	4.249	0.046
Propionic acid(mmol/L)	40.34	27.16	4.493	0.018
Butyric acid(mmol/L)	8.00	10.24	0.854	0.022
Total volatile fatty acid(mmol/L)	107.98	106.47	6.413	0.819
Acetic acid/ propionic acid	1.58	2.64	0.337	0.008
NH_3-N/(mg/dL)	13.24	13.05	1.93	0.921

[1]bpm = breath per minute.

Table 2 Comparison of metabolite content of rumen fluid between heat-sensitive (HS) cows and heat-tolerant (HT) cows

Name	VIP[1]	*P*-value	Log_2FC[2]	KEGG ID	KEGG[3] pathway	Tendency
Mannitol	1.12	0.02	3.60	ko02010	Carbohydrate metabolism; Membrane transport	↑
Glycerol	2.24	0.02	2.69	ko01100	Carbohydrate metabolism; Lipid metabolism; Membrane transport; Nervous system	↑
Maltose	1.05	0.00	1.43	ko01100	Carbohydrate metabolism; Lipid metabolism; Membrane transport; Nervous system	↑

[1]VIP = variable important in projection.

[2]Log_2FC = fold changes (FC) of the concentrations of metabolite where log transformed as 2-log of FC.

[3]KEGG = Kyoto Encyclopedia of Genes and Genomes.

[4]Tendency "↑" = higher abundance in HT cows.

Figures

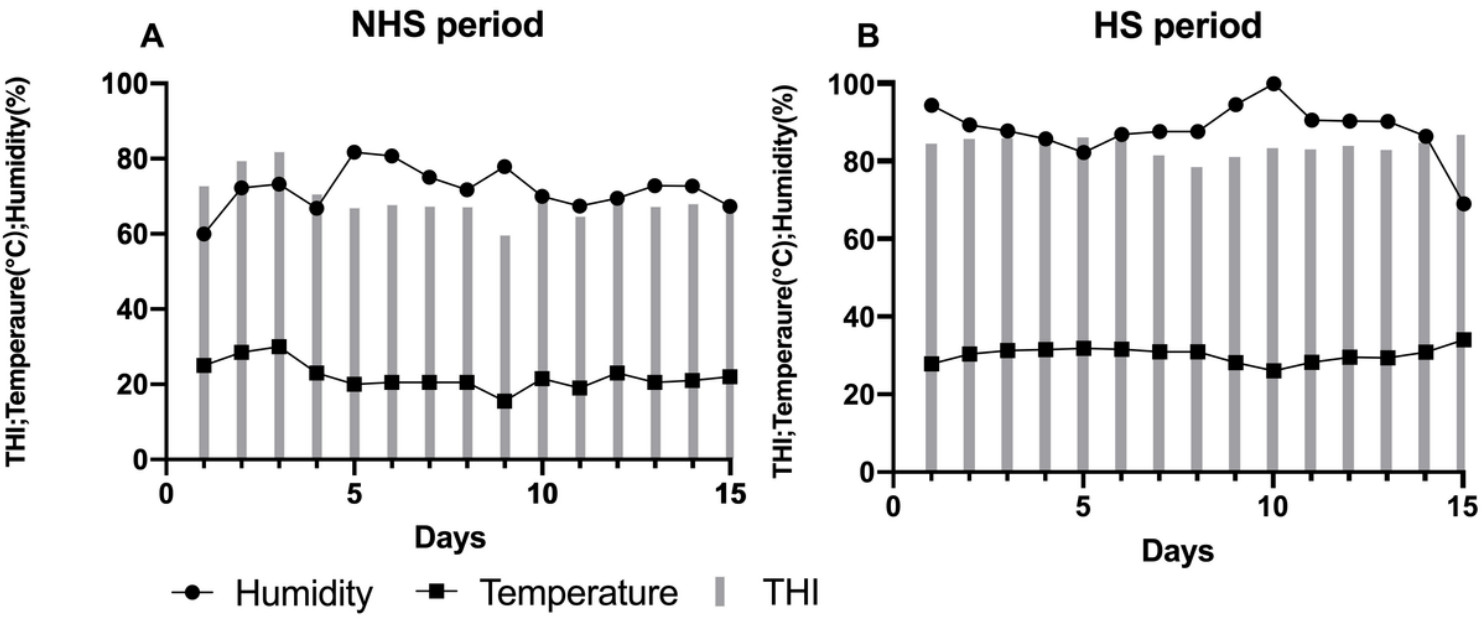

Figure 1

Temperature, humidity, and THI values (mean/d) during different periods. a Temperature, humidity, and THI values during non-heat stressed period of May (NHS period). b Temperature, humidity, and THI values

during heat-stressed period of July (HS period).

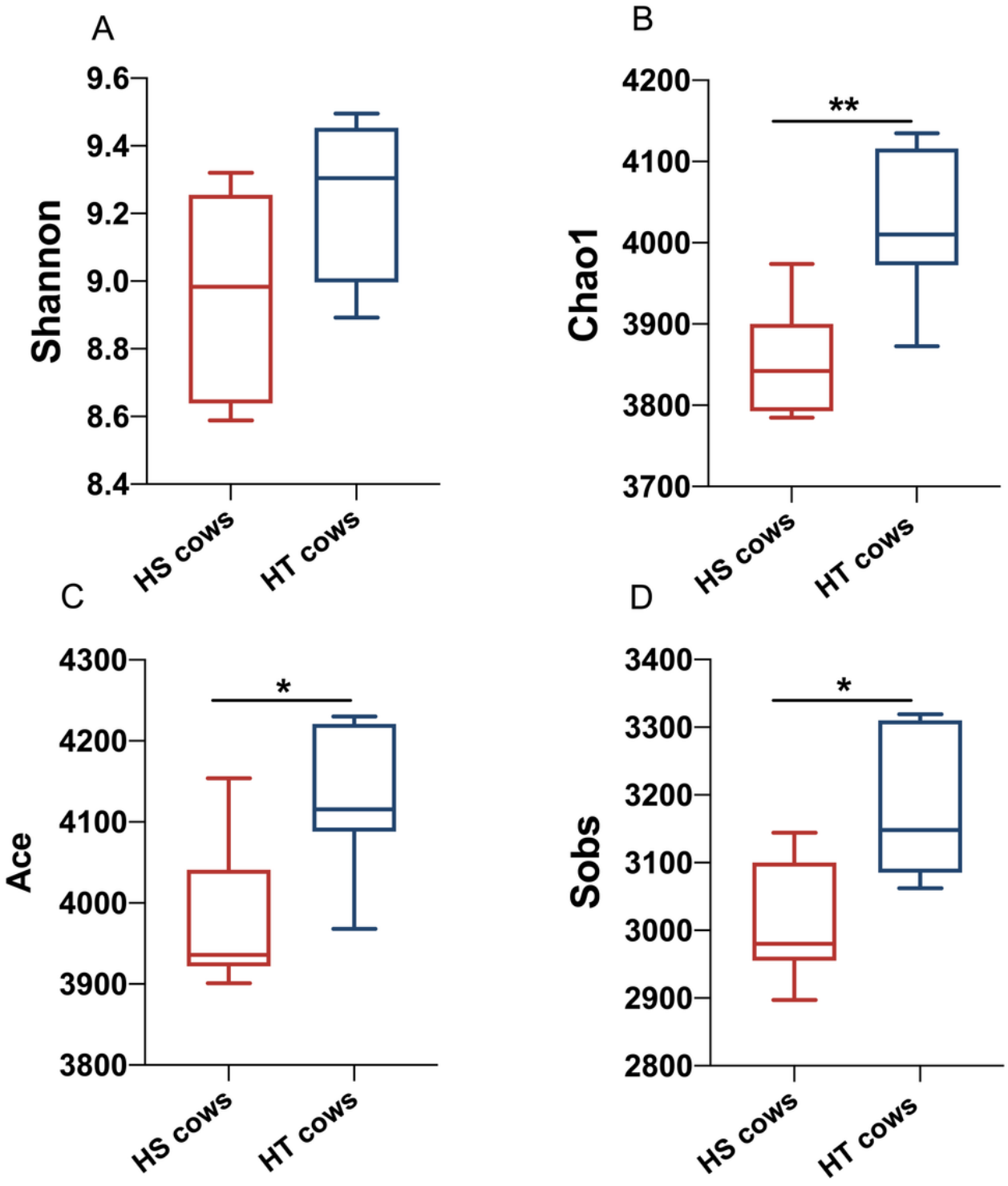

Figure 2

Analysis of rumen microbial alpha diversity in heat-tolerant (HT) and heat-sensitive (HS) cows. Shannon (a) was the diversity indices. Chao 1 (b) and Ace (c) were the richness estimators. Sobs (d) was the actual numbers of OTUs observed.

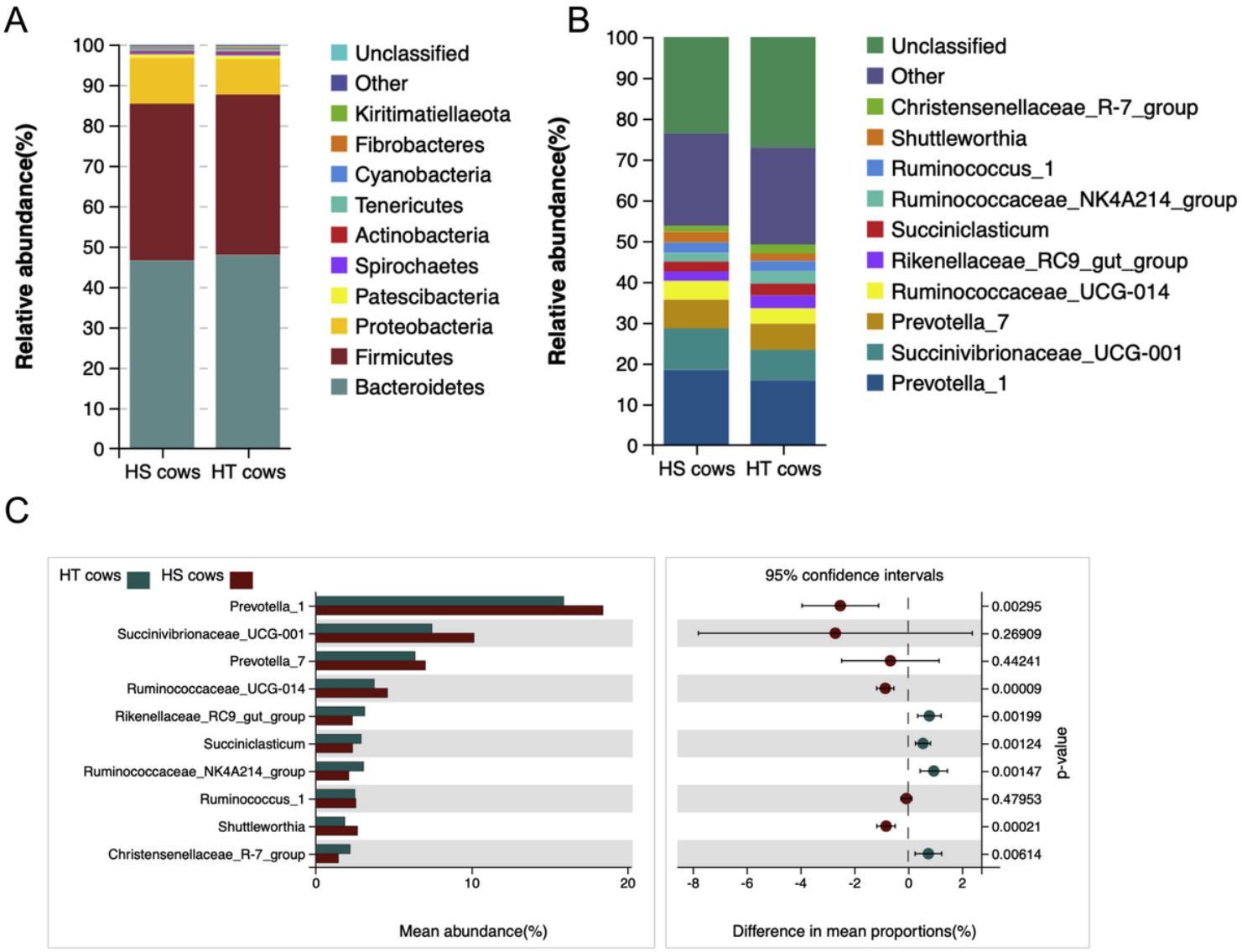

Figure 3

Microbial compositional profiles of heat-sensitive (HS) and heat-tolerant (HT) cows at phylum and genus levels. The relative abundance (%) of rumen microorganisms at the phylum (a) and genus (b) levels between HT and HS cows. Species difference analysis of rumen microorganisms at the genus (c) levels between HT and HS cows.

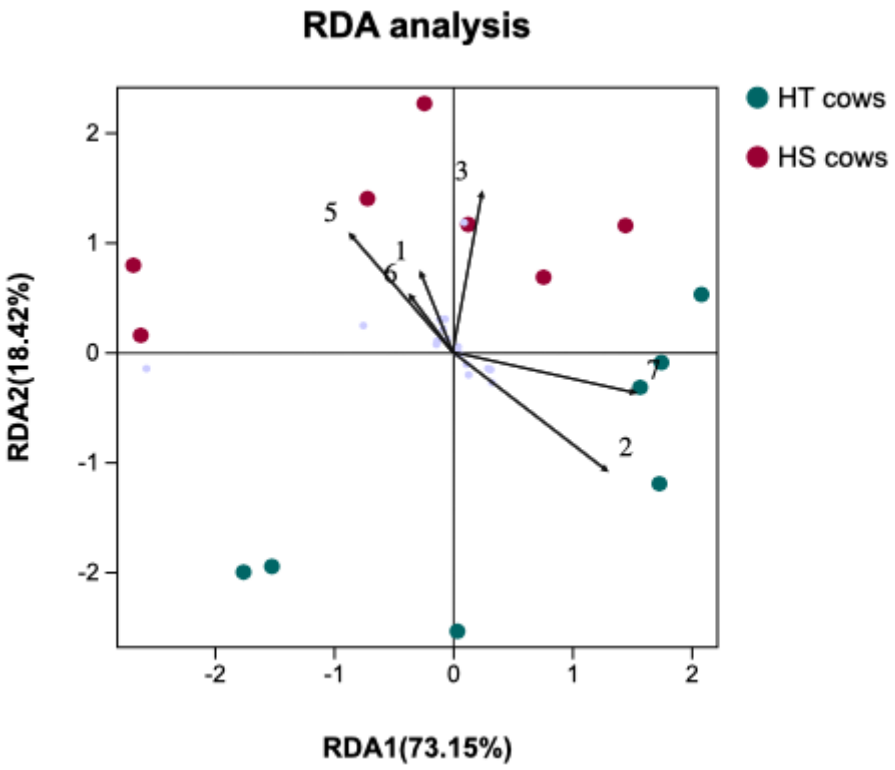

Figure 4

Redundancy analysis (RDA) of microbial community composition difference in relation to rumen fermentation parameters and heat tolerance indexes. Arrows 1-7 represent acetic acid, propionic acid, butyric acid, total volatile fatty acids, acetate/propionate, pH, and ammonia nitrogen, respectively. HT cows = heat-tolerant; HS cows = heat-sensitive cows

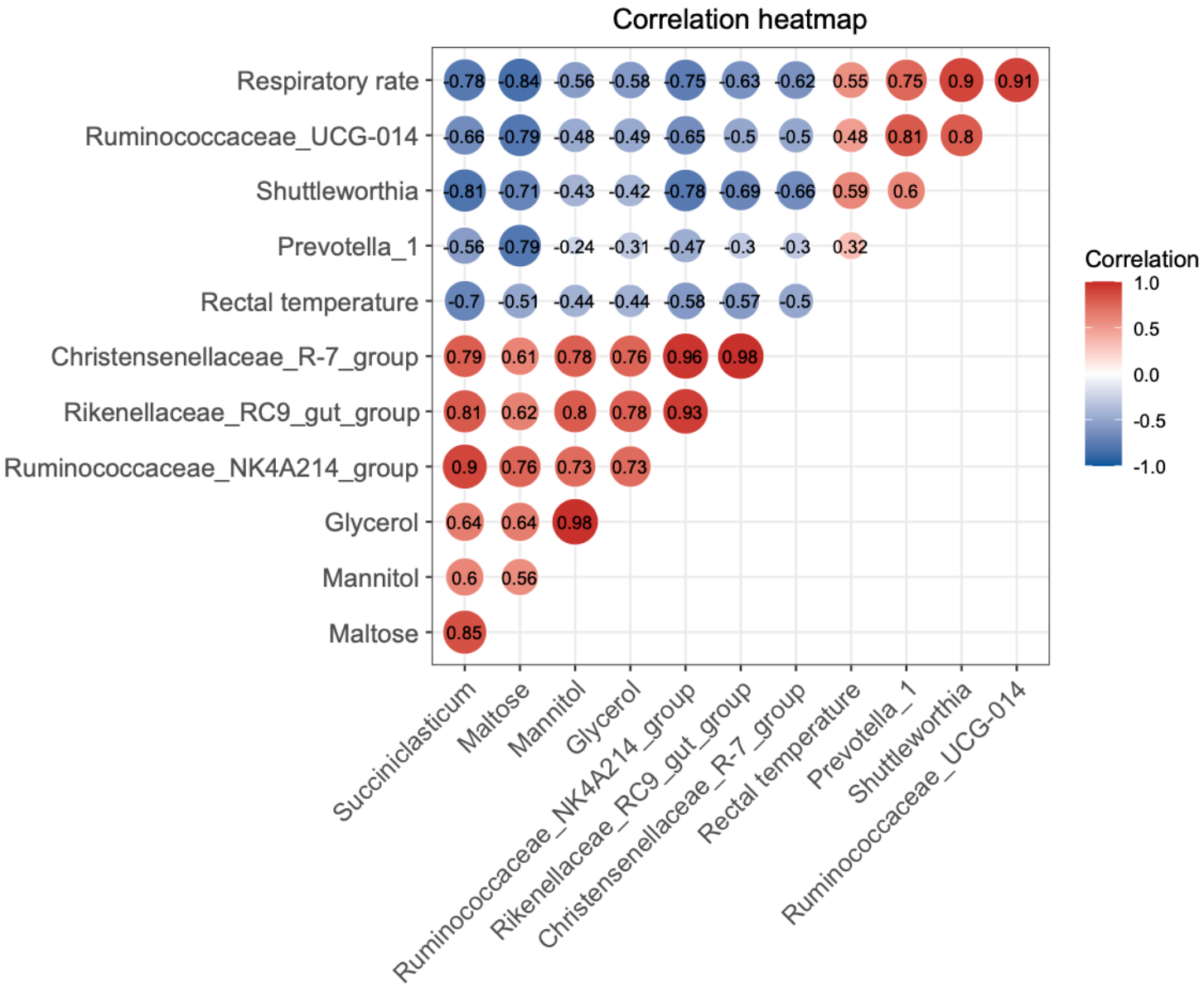

Figure 5

Relationships between heat tolerance indexes, rumen fermentation parameters, and metabolites.

Supplementary Files

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