Animal Microbiome

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Cooling redistributed endotoxin across different biofluids via modulating the ruminal microbiota and metabolome without altering quorum sensing signal levels in heat-stressed beef bulls

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Abstract

Background Cooling is one of the most common and economical methods to ameliorate heat stress (HS), and it has been discovered to alter the lipopolysaccharide (LPS) endotoxin level in ruminants. However, whether the endotoxin variation induced by cooling relates to the quorum sensing (QS) within the ruminal microflora remains unknown. The current study was consequently performed to examine whether cooling could influence the endotoxin distribution across different biofluids, ruminal microbiota, and ruminal metabolisms through affecting the QS of rumen microorganisms in beef cattle exposed to HS. Thirty-two Simmental bulls were used as experimental animals and randomly assigned to either the control (CON) group, or the mechanical ventilation and water spray (MVWS) treatment. The temperature-humidity index (THI) was recorded throughout this trial, and samples of the rumen liquid, blood, and urine were collected.

Results Cooling significantly lowered ($P < 0.05$) the temperature-humidity index (THI), ruminal endotoxin, and endotoxin concentration and excretion in urine, and significantly raised endotoxin level in blood ($P < 0.05$), but did not change the ruminal concentrations of QS signals including 3-OXO-C6-HSL and the AI-2 ($P > 0.05$). The linear discriminant analysis effect size (LEfSe) analysis revealed that *Prevotellaceae*, *Rikenellaceae*, Monoglobales and their affiliated members, as well as other bacterial taxa were significantly differently ($P < 0.05$) enriched between the two treatments. The Tax4Fun2 prediction suggested that QS function was upregulated in MVWS compared to CON. The metabolomic analysis indicated that cooling altered the ruminal metabolism profile and downregulated the pathways of lysine degradation, phenylalanine, tyrosine and tryptophan biosynthesis, and ubiquinone and other terpenoid-quinone biosynthesis. The significant ($P < 0.05$) correlations of the differential bacteria and metabolites with endotoxin and QS molecules were also demonstrated through Spearman analysis.

Conclusions Based on the results of this trial, it could be speculated that the cooling reshaped the endotoxin distribution across different biofluids through manipulating ruminal microbiota and metabolome, which might involve

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the participation of QS. Further investigations are warranted to disclose and verify the mechanisms for those correlations found in this study.

Keywords Heat stress, Endotoxin, Quorum sensing, Rumen microbiome, Cooling

Background

The climate warming has been posing an increasingly stern challenge to global ruminant husbandry [1], since the ruminants are susceptible to the heat stress (HS) caused by the deteriorations of multiple environmental factors including the ambient temperature, relative humidity, thermal radiation, and air movement [2]. It has been commonly acknowledged that, as a critical environmental stressor, HS would impair both the production and reproduction performances of domestic ruminants [3, 4]. In particular, Wang et al. [5] reported the rise in the flux of bacterial lipopolysaccharide (LPS) endotoxin across the portal-drained and mesenteric-drained viscera under HS condition, which could successively lead to the expression of pro-inflammatory cytokines and the systematic inflammation, as HS injures the barrier integrity of gastro-intestinal tract of ruminants [6, 7].

A few studies including our precedent investigations have revealed that cooling (a major method of thermal climate management) [8], as well as the nutritional interventions such as raising dietary cation–anion difference [6] and supplementing L-theanine [9] both altered endotoxin concentrations in different biofluids (i.e., rumen fluid, blood, milk, and urine) and attenuated relevant inflammatory responses in dairy cows subjected to HS. Nevertheless, although mitigation strategies targeting HS and their effects have been intensively investigated in dairy production system, information on the solutions for ameliorating HS of beef cattle is somewhat lacking [10].

Quorum sensing (QS) is a universal cell-to-cell communication mode also possessed by the ruminal microbiome [11, 12], and its regulatory role in the LPS endotoxin production of some typical gram-negative bacteria has been verified [13]. However, whether the HS mitigation strategies that have been found to modulate the endotoxin level in the rumen fluid could influence QS within the ruminal bacterial populations is still uncertain. Therefore, the present study was performed to explore the impacts of cooling by mechanical ventilation and water spray on the endotoxin contents across multiple biofluids (i.e., rumen fluid, blood, and urine) and the QS signaling molecules, together with the reactions of the ruminal bacterial microflora and ruminal metabolisms in beef cattle.

Methods

Animals, management, and diet

Regarding all the relevant animal procedures, this study was conducted with the supervision and approvement from the Animal Care Committee (approval number: 20220626), College of Animal Science and Technology, Hunan Agricultural University, Changsha, China. The current trial was carried out from the July to August of 2022 at a beef cattle farm in Huaihua, China. Thirty-two healthy Simmental bulls averaging 420 ± 47 kg (initial mean ± SD) of body weight were used as the experimental animal following a completely randomized design. All the bulls were randomly assigned to either the control (CON) group or the mechanical ventilation and water spray (MVWS) treatment. Cattle of these two treatments were separately housed in two tie-stall barns, and the MVWS barn was equipped with fans and sprinklers. More specifically, a total of 6 fans (motor speed: 710 rpm) were uniformly set on the both sides of the MVWS barn, with the height at 2.5 m, an interval of 6 m, and an angle at 60° to the horizontal. A sum of 52 sprinklers (each water flow: 0.02–0.05 L/min) were evenly installed at the height of 1.7 m and the interval of 1.5 m. The fans and sprinklers worked constantly from 08:00 to 20:00 on each day, and automatically paused if the temperature in the barn dropped below 25 °C. Bulls from the two treatments were fed the same basal total mixed ration (TMR) diet (Table 1) ad libitum twice (06:00 and 18:00) per day, and provided with free access to fresh water. The whole experiment consisted of 15 days of adjustment and 30 days of data and sample collection.

Data and sample collection

Throughout the 30 days for collecting data and samples, the temperature-humidity index (THI) value was calculated and recorded thrice (08:00, 14:00, and 20:00) daily as previously described [9], using 12 thermo-hygrometers (9013, Deli Group, Ningbo, China) equably placed in the two barns at the height of 1.5 m. Cattle were considered as being exposed to HS when the THI rose above the threshold of 72 [14]. The collection and preparation

Table 1 Constituents and nutritional composition of the basal TMR ration

Ingredient[1], g/kg DM		Nutrient content[2], g/kg DM	
Pennisetum purpureum	345	NE_{mf}, MJ/kg	5.26
Peanut stem	141	OM	901.1
Stevia rebaudiana root	128	CP	117.2
Corn meal	229	NDF	386.2
Soybean meal	92	ADF	245.3
DDGS	26	EE	11.7
Wheat bran	22	Ash	98.9
$CaHCO_3$	10	Ca	20.3
NaCl	5	P	7
Premix	2		

[1] DDGS, distillers' dried grains with soluble; Every 1 kg of premix comprises 4,500,000 IU of vitamin A, 300,000 IU of vitamin D3, 6000 IU of vitamin E, 10.5 mg of Zn, 15.3 g of Mn, 23.2 g of Fe, 8.6 g of Cu

[2] NE_{mf}, combined net energy, calculated according to the China Feeding Standard of Beef Cattle (NY/T815-2004); OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extract

of samples from the rumen fluid, blood, and urine was performed using sterilized tools on the day 30 and 45, in accordance to the operations detailedly illustrated in our precedent report [6]. Additionally, the rumen liquid sampled 2 h before 4 h after morning feeding were equally mixed. For the full-length 16S rRNA gene sequencing and metabolomic analysis, six bulls from each treatment were randomly chosen and their rumen fluid samples were thence used. All the above samples were preserved at − 20 °C until the subsequent analysis. The data for production performance, rumen fermentation characteristics, and physiological indexes of the bulls in this experiment has been published elsewhere [15].

Chemical and biochemical analysis

The nutritional contents of the basal TMR diet were analyzed as priorly depicted [16]. For the measurement of LPS endotoxin in the rumen liquid, blood, and urine, the chromogenic endpoint Tachypleus Amebocyte Lysate assay kit (EC80545S, Chinese Horseshoe Crab Reagent Manufactory Co. Ltd, Xiamen, China) was employed referring to our previously introduced procedures [6, 9]. The urine volume of each cattle was estimated by employing urinary creatinine as the marker [17, 18], and it was further used to assess the daily discharge of endotoxin via the urine.

The signaling molecule autoinducer-2 (AI-2) of QS was detected on a 1260 high performance liquid chromatography equipped with a fluorescence detector (HPLC-FD) system (Agilent, Santa Clara, USA) using a ZORBAX Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm) (Agilent, Santa Clara, USA), by following the general approaches developed by Song et al. [19]. As to the QS signals of acyl-homoserine lactones (AHLs), the quantification was conducted on the ExionLC 30A-QTRAP 5500 ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) system (AB SCIEX, Framingham, USA) with an ACQUITY BEH C18 column (2.1 × 100 mm) (Waters, Milford, USA), as depicted in previous study [20].

Full-length 16S rRNA gene sequencing analysis

The genomic DNA was firstly extracted from the rumen fluids by adopting the phenol-free bead-beating method introduced by Yu and Morrison [21], and was subsequently used to amplify the full-length 16S rRNA genes through the universal primers 27F (50-AGRGTTTGATYNTGGCTCAG-30) and 1492R (50-TASGGHTACCTTGTTASGACTT-30) with barcodes. The amplicon sequencing was performed on the PacBio Sequel II platform (Pacific Biosciences, Menlo Park, United States) with single-end reads generated. The raw sequencing data pretreatment consisted of the circular consensus sequencing (CCS) reads recognition, CCS reads quality filtering, and chimera sequence removal was conducted by referring to approaches depicted in our precedent studies [22]. The operational taxonomic unit (OTU) taxonomic annotation was carried out using the SILVA database (release 138) with a confidence threshold at 80%, and OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the fewest sequences. The analysis for Alpha diversity and Beta diversity were performed through the QIIME (version 1.9.1) and R software (version 3.5.0), and Tax4Fun2 (version 1.1.5) function prediction were consecutively operated complying with the methods adopted in our prior investigations [16, 22, 23]. All the raw sequences generated in this study were submitted to the sequence read archive (SRA) of the NCBI database under the accession number PRJNA1066970.

Metabolomics analysis

The metabolomic analysis for the rumen liquid samples was fulfilled through the successive processes of the metabolites isolation, UHPLC-MS analysis, raw data treatment, metabolites classification, and metabolomics data analysis, by adopting our previous approaches [24]. Metabolites respectively identified under the positive and

negative polarity mode were combined, and the principal components analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) were performed following the internal standard normalization and data logarithmic conversion. Annotation for the metabolic pathways and relevant enrichment analysis were accomplished using the MetaboAnalyst (version 5.0) by referring to the Kyoto encyclopedia of genes and genomes (KEGG) database (release 110.1) [26].

Statistical and data analysis

To evaluate the effects of cooling on the endotoxin concentration and excretion, QS signaling molecules, and Alpha diversity indices, the corresponding data were analyzed using SPSS statistics (version 23.0, IBM Corporation, Armonk, United States) through the unpaired t-test, whilst the THI index data recorded during this trial were analyzed by adopting the GLMM model. Statistical difference was considered as significant, highly or extremely significant at the adjusted $P < 0.05, < 0.01$ or < 0.001, respectively. The linear discriminant analysis effect size (LEfSe) was used to contrast the relative abundances of microbial taxa between the two treatments, and significant differences were declared with a linear discriminant analysis (LDA) score > 3 and adjusted $P < 0.05$. The differential predicted functions and differential metabolites between treatments were examined through the rank-sum Wilcoxon test, and differential metabolites were further annotated as significantly different with the variable importance in projection (VIP) value > 1 and adjusted $P < 0.05$. The Spearman correlation analysis was employed to check the interconnections between endotoxin, QS signals, bacterial taxa (the overall 18 phyla, the top 30 genera and species), and significantly differential metabolites. The model-based integration of metabolite observations and species abundances 2 (MIMOSA2, version 2.1.0) [25] analysis was performed by combining the data of 16S rRNA gene sequencing and metabolites annotation in the present investigation.

Results

THI, endotoxin, and QS signals

Throughout the entire period of data and sample collection, even though the most majority of the average daily THI indexes recorded in both of the two barns exceeded the threshold at 72, the averaged THI value in MVWS was significantly lower than that in the CON ($P < 0.05$) (Fig. 1A). Besides, cooling highly significantly or significantly decreased the LPS endotoxin densities in the rumen fluid ($P < 0.01$) and urine ($P < 0.01$), as well as the urinary excretion of endotoxin ($P < 0.05$) (Fig. 1B–E). Nonetheless, the endotoxin concentration in the blood of MVWS bulls was extremely higher than their counterparts in the CON ($P < 0.001$). The 3-OXO-C6-HSL and the AI-2 were the only two QS signaling molecules detected in the rumen fluid of this trial, and significant difference was observed in none of these two molecules between the two treatments ($P > 0.05$) (Fig. 1F–G).

Ruminal bacterial community

In the current trial, cooling was found to exert profound influences on the entire ruminal bacterial microbiota. It was firstly revealed that none of the Alpha diversity indexes for the ruminal bacterial microbiota in the heat-stressed bulls was influenced by cooling ($P > 0.05$) (Fig. 2A–D, Table S1), and no treatment-dependent clustering of the bacterial populations was presented through the either the unweighted Unifrac-based or the weighted Unifrac-based Principal coordinate analysis (PCoA) analysis (Fig. 2E–F). According to the LEfSe diagram showing the significantly differential taxa (Fig. 2G–H, Tables S2, S3, S4), the bacterial family *Prevotellaceae* and its sequentially subordinate genera of *Prevotella* and *Prevotellaceae UCG-001*, as well as species of *uncultured_rumen_bacterium_g_Prevotella* and *uncultured_rumen_bacterium_g_Prevotellaceae_UCG-001* were significantly enriched in the CON ($P < 0.05$), whilst the *uncultured_rumen_bacterium_g_Sphaerochaeta*, *unclassified_g_Lachnospiraceae_UCG-006*, *unidentified_rumen_bacterium_RFN85*, and *unclassified_g_Acetitomaculum* were also identified with significantly higher abundances in CON compared to MVWS ($P < 0.05$). In comparison, the species of *uncultured_bacterium_g_Rikenellaceae_RC9_gut_group* and *uncultured_rumen_bacterium_g_Rikenellaceae_RC9_gut_group*, the genera *Rikenellaceae RC9_gut_group*, and their upper family *Rikenellaceae* were successively significantly enriched in MVWS ($P < 0.05$). Besides, the relative abundances of Monoglobales, *Monoglobaceae*, and *Monoglobus* were significantly increased in MVWS compared with the CON ($P < 0.05$), from the order to the genus level. Moreover, the order no_rank_c_Clostridia, the genus *UCG-004*, and the species *unclassified_g_Christensenellaceae_R-7_group* were also discovered with significantly more quantities in MVWS ($P < 0.05$).

The Spearman analysis suggested that the phylum WPS-2 was significantly positively ($P < 0.05$) related to the endotoxin level in blood and 3-OXO-C6-HSL in rumen liquid, and Planctomycetota was significantly positively ($P < 0.05$) connected to the AI-2 (Fig. 3A). At the genus level, a significantly positive relation ($P < 0.05$) between the *norank_f_Bacteroidales_BS11_gut_group*

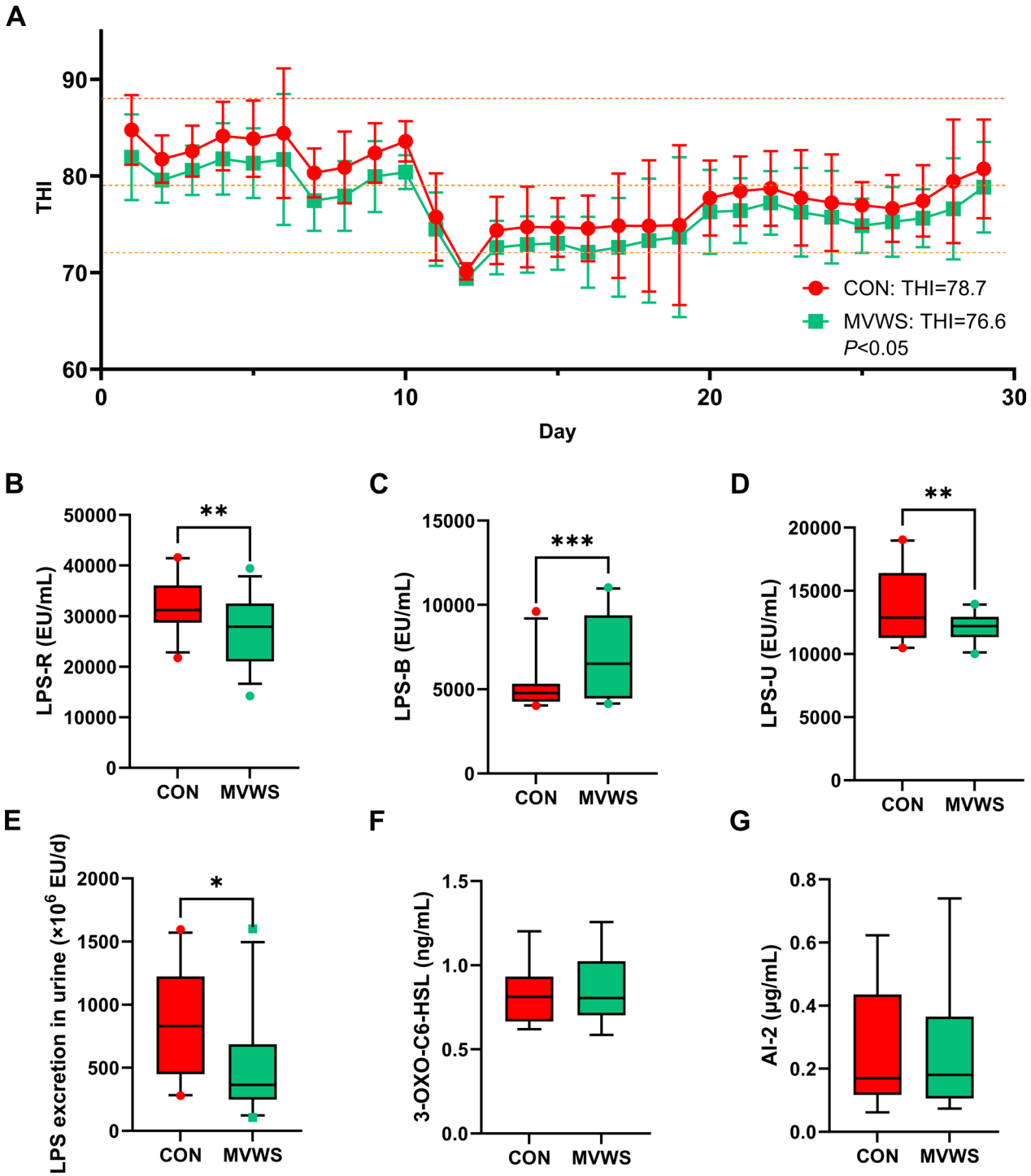

Fig. 1 Effects of cooling on the THI index, endotoxin, and QS signaling molecules. *: significant ($P < 0.05$); **: highly significant ($P < 0.01$); ***: extremely significant ($P < 0.001$). **A** Averaged daily THI values in the two treatment barns throughout the period for data and sample collection, **B** LPS endotoxin concentration in the rumen fluid. **C** LPS endotoxin concentration in the blood, **D** LPS endotoxin concentration in the urine, **E** estimated LPS endotoxin excretion through the urine, **F** 3-OXO-C6-HSL belonging to the AHLs. **G** AI-2

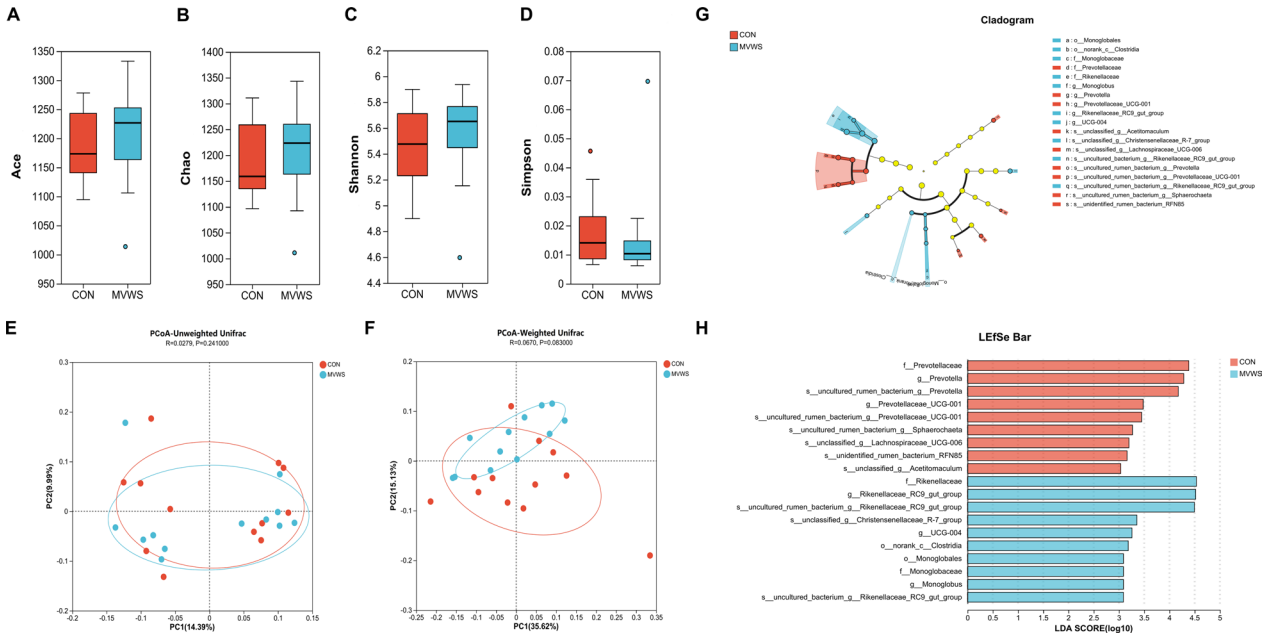

Fig. 2 Effects of cooling on Alpha diversity indices, Beta diversity, and bacterial taxa relative abundances of the ruminal bacterial microbiota. **A** Ace index, **B** chao index, **C** Shannon index, **D** Simpson index, **E** principal coordinate analysis (PCoA) profile based on the unweighted Unifrac matrix, **F** PCoA profile based on the weighted Unifrac matrix, **G** Cladogram displaying significantly enriched bacterial taxa (LDA score > 3.0 and $P < 0.05$) from the phylum to the species level through the LEfSe analysis. Red: taxa abundant in CON; Blue: taxa abundant in MVWS, **H** LEfSe bar chat displaying the significantly differential taxa between treatments. The LDA scores represented the difference in relative abundance with exponent fold change of 10 across treatments

and the endotoxin excretion in urine was marked, while the significantly negative and positive relationship ($P < 0.05$) between *Anaeroplasma* and endotoxin in blood and its discharge through urine were observed, respectively (Fig. 3B). The *norank_f_Lachnospiraceae* was found significantly positively ($P < 0.05$) related to blood endotoxin concentration, and *Eubacterium_hallii_group* was significantly positively ($P < 0.05$) connected to the urinary endotoxin level. A significantly negative connection between *UCG-002* and 3-OXO-C6-HSL, as well as the significantly positive relationships between AI-2 and bacterial phyla including *Sphaerochaeta*, *norank_f_p-2534-18B5_gut_group*, *Acetitomaculum*, and *p-1088-a5_gut_group* were exhibited. In terms of the bacterial species, the *Ruminococcus_sp._g_Ruminococcus* and *uncultured_rumen_bacterium_g_Eubacterium_hallii_group* were both significantly positively ($P < 0.05$) related to the urinary endotoxin, whilst *uncultured_rumen_bacterium_g_Anaerovorax* was significantly negatively ($P < 0.05$) connected to the endotoxin content in urine (Fig. 3C). The molecule 3-OXO-C6-HSL was significantly negatively ($P < 0.05$) correlated to *uncultured_rumen_bacterium_g_UCG-002*. As to the AI-2, its significantly positive ($P < 0.05$) correlations with *uncultured_rumen_bacterium_g_norank_f_p-2534-18B5_gut_group* and *unclassified_g_Lachnospiraceae_NK3A20_group*, and significantly negative ($P < 0.05$) connections with *uncultured_rumen_bacterium_g_Prevotellaceae_UCG-003* and *unclassified_g_Prevotella* were illustrated, respectively.

As was illustrated through the Tax4Fun2 prediction with high accuracy, a sum of 19 potential functions of the ruminal bacterial microbiome were classified as significantly differentially expressed between the CON and MVWS (Fig. 3D). The relative abundances of bacteria participating in metabolic pathways, riboflavin metabolism, ubiquinone and other terpenoid-quinone biosynthesis, and other 10 functions in the CON were significantly higher ($P < 0.05$) than those in MVWS. By contrast, the QS, bacterial chemotaxis, flagellar assembly, histidine metabolism, C5-branched dibasic acid metabolism, and salmonella infection were predicted to be significantly upregulated ($P < 0.05$) in the rumen bacteria of the MVWS cattle.

Ruminal metabolome

The effects of cooling on the ruminal metabolisms of the heat stressed bulls were demonstrated through the

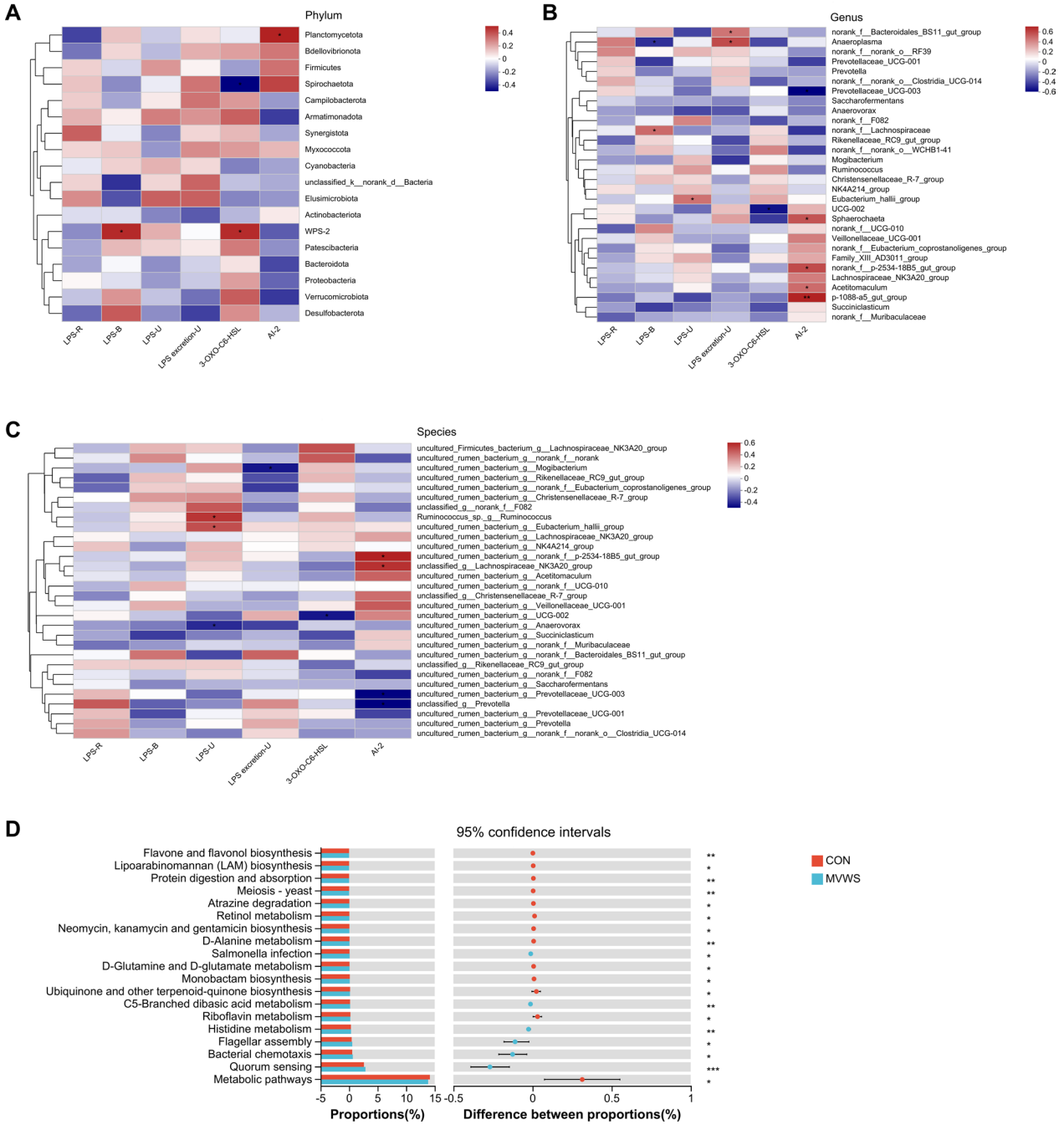

Fig. 3 Correlations between endotoxin, QS signals, and bacterial taxa, and the Tax4Fun2 prediction for the ruminal bacterial populations. **A** Correlations between endotoxin, QS signals, and bacterial taxa at the phylum level. Red: positive; Blue: negative, **B** correlations between endotoxin, QS signals, and bacterial taxa at the genus level, **C** correlations between endotoxin, QS signals, and bacterial taxa at the species level, **D** Wilcoxon test for the predicted potential functions for the ruminal bacterial microflora between treatments based on the Tax4Fun2 analysis

metabolomic analysis of this study. Although no separation in the metabolic profiles between the two treatments was depicted in the PCA chart (Fig. 4A), the OPLS-DA score diagram suggested a clear treatment-dependent clustering of the metabolite profile of rumen fluid (Fig. 4C), with its model validity confirmed by the permutation test (Fig. 4B). It was further revealed that 31 metabolites amongst the 710 totally detected in this trial (Table S5) were classified as significantly different ($P < 0.05$) in the comparison of MVWS to CON, within

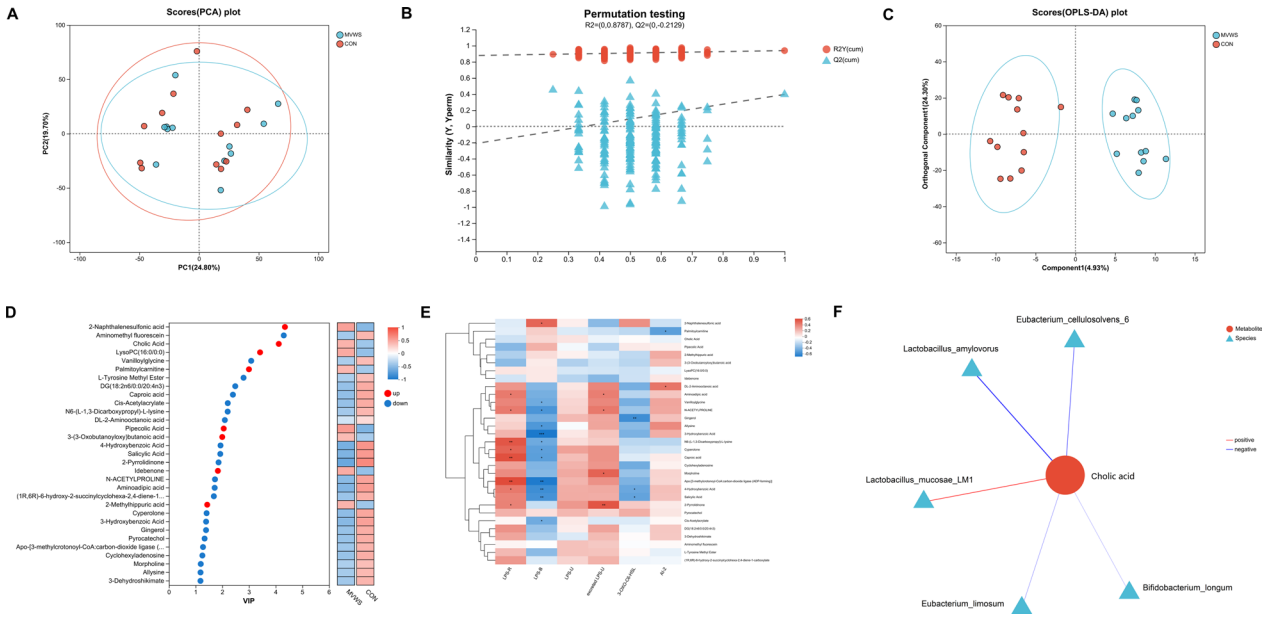

Fig. 4 Effects of cooling on the profile of the ruminal metabolites, and the correlations between endotoxin, QS signals, differential metabolites, and bacterial species. **A** Principal component analysis (PCA) plot, **B** permutation test plot of the orthogonal projections to latent structures-discriminant analysis (OPLS-DA), **C** OPLS-DA score scatter plot, **D** variable importance in projection (VIP) bubble plot for the differential metabolites, **E** correlations between endotoxin, QS signals, and the differential metabolites, **F** model-based integration of metabolite observations and species abundances 2 (MIMOSA2) analysis illustrating the potential correlations between the bacterial species and the differential metabolites

which the 2-naphthalenesulfonic acid, cholic acid, and other 6 metabolites were upregulated, while aminomethyl fluorescein, vanilloylglycine, and other 19 metabolites were downregulated (Fig. 4D, Table S6).

A considerable portion of the differential metabolites decreased in MVWS, e.g., the N-acetylproline, caproic acid, and 4-hydroxybenzoic acid, were discovered being significantly negatively ($P<0.05$) connected to the blood endotoxin (Fig. 4E), whereas the significantly positive ($P<0.05$) correlations between some of these downregulated metabolites and the endotoxin in rumen fluid and its discharge through urine were noticed. In addition, gingerol, 4-hydroxybenzoic acid, and salicylic acid were significantly positively ($P<0.05$) related to 3-OXO-C6-HSL, and these metabolites were all reduced in MVWS. Furthermore, the increased palmitoylcarnitine and the decreased DL-2-aminooctanoic acid in MVWS were found to be significantly negatively and positively ($P<0.05$) interrelated to AI-2, respectively.

As was portrayed through the MIMOSA2 analysis based on the combination of 16S rRNA gene sequencing and metabolomics analysis (Fig. 4F), the species *Lactobacillus mucosae_LM1* positively contributed to the increment of cholic acid in MVWS, while the negative connections between cholic acid and *Lactobacillus amylovorus*, *Eubacterium cellulosolvens_6*, *Eubacterium limosum*, and *Bifidobacterium longum* were also shown.

Based on the KEGG enrichment analysis, a total of 10 metabolic pathways were identified as differential pathways between the two treatments (Fig. 5A, Table S7). It was further manifested by the differential abundance score plot that amongst the above 10 differential pathways, the lysine degradation, phenylalanine, tyrosine and tryptophan biosynthesis, and ubiquinone and other terpenoid-quinone biosynthesis were marked as extremely significantly ($P<0.001$), highly significantly ($P<0.01$), and significantly ($P<0.05$) downregulated in MVWS compared with CON, respectively (Fig. 5B). Besides, the significant ($P<0.05$) upregulation of bile secretion and choline metabolism in cancer in MVWS was also observed. By referring to the reference map of the KEGG database, the significantly different pathways of phenylalanine, tyrosine and tryptophan biosynthesis, and ubiquinone and other terpenoid-quinone biosynthesis were further integrated, with the involving differential metabolites exhibited alongside (Fig. 5C).

Discussion

The present study was designed to investigate whether cooling, the primary anti-HS strategy, could change the endotoxin distribution via influencing the bacterial QS function as well as the overall ruminal bacterial microbiome and metabolome. It was firstly observed that, despite the fact that the overwhelming majority of the daily THI

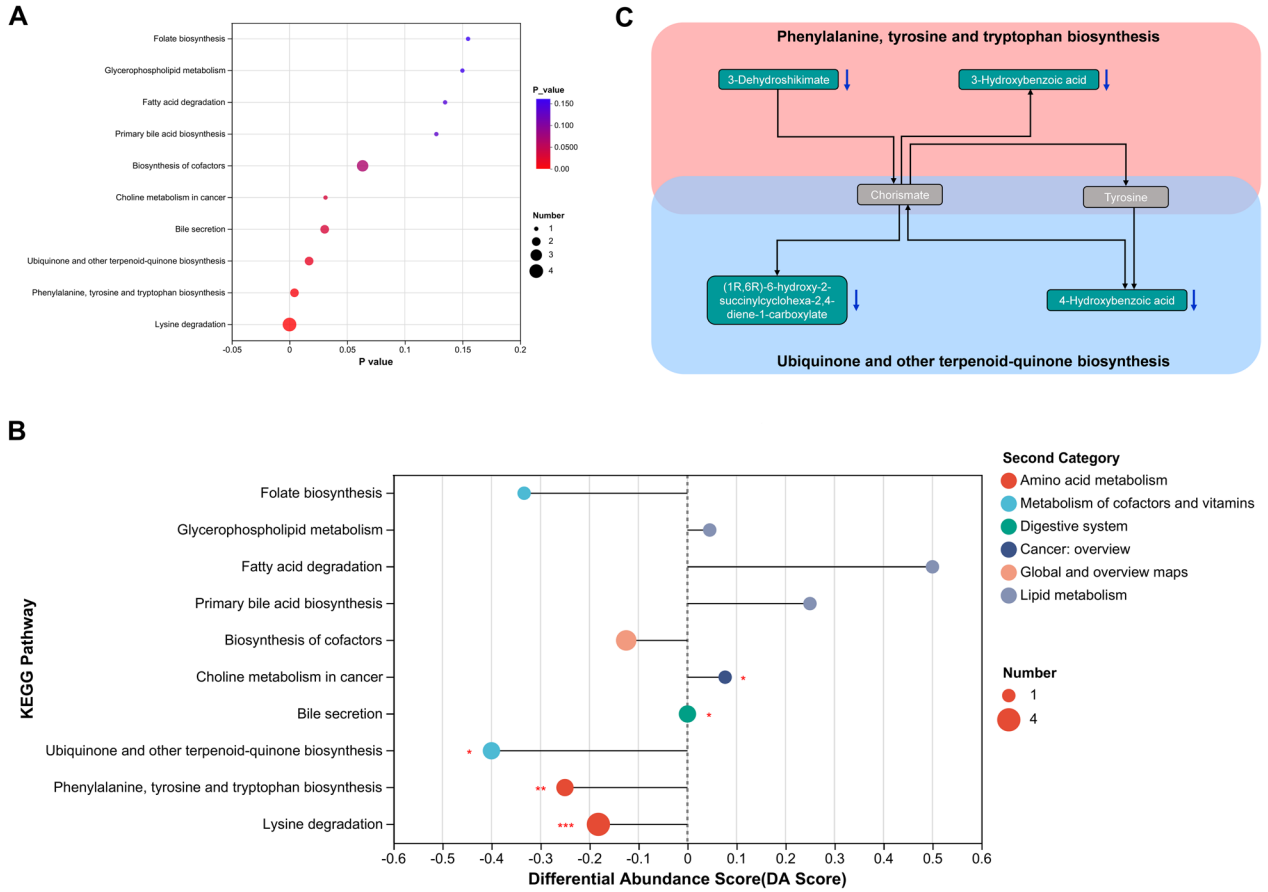

Fig. 5 Effects of cooling on the ruminal metabolomic pathways. **A** Bubble plot for the KEGG enrichment analysis of the differential metabolomic pathways between treatments. The size of the bubble represents the number of the enriched differential metabolites for each pathway, **B** differential abundance score plot for the differential metabolomic pathways in the comparison of MVWS to CON. The size of the bubble represents the number of the enriched differential metabolites for each pathway, **C** integrated significantly differential metabolic pathways between treatments. The down arrow indicates that the metabolite was significantly downregulated ($P < 0.05$) in the comparison of MVWS to CON

values of the both two barns were beyond the mild HS threshold at 72 [14], the averaged THI of the CON barn exceeded the threshold of high HS at 78 [27], whilst the averaged THI of the MVWS barn fell below that value. This discrepancy indicated that the bulls receiving cooling were exposed to less severe HS throughout this trial, testifying the apparent mitigatory effects of cooling as one of the most practicable and efficient strategies against HS [28]. Unlike the L-theanine supplementation at 16 g/d that raised the LPS endotoxin level in the rumen fluid of heat-stressed dairy cows in our prior investigation [9], the decrement of the ruminal endotoxin by cooling was noticed in the present experiment, which implied that the endotoxin production of the ruminal gram-negative bacteria could be reduced. However, it was also surprisingly noted that the blood endotoxin content in MVWS were higher than the CON, which was yet in agreement with the previous finding that cooling increased endotoxin level in the blood of dairy cows under HS [8]. Further in this study, both the concentration and elimination of endotoxin in the urine declined in response to cooling, which might partially lead to the above-mentioned increasing accumulation of endotoxin in the peripheral blood. This speculation requires deeper investigations to be examined.

In the current trial, cooling seemed to trigger the variations in the abundances of several bacterial taxa at different levels without altering the QS signals, as well as the Alpha and Beta diversity of the bacterial community in rumen liquid. It was noteworthy that the family *Prevotellaceae* and its subordinates (i.e., *Prevotella*, *Prevotellaceae_UCG-001*, *uncultured_rumen_bacterium_g_Prevotella*, and *uncultured_rumen_bacterium_g_Prevotellaceae_UCG-001*) were reduced in MVWS, when compared with the CON. These *Prevotellaceae* members have been found to contribute to less weight gain of goat, mice, and human [29–31]. In fact, the bulls treated with cooling of this experiment tended

to show greater average daily gain (ADG) [15], which was consistent with the reductions of the above *Prevotellaceae*-related taxa in MVWS group. As a negative correlation between the genus *Lachnospiraceae UCG-006* and the butyric acid concentration has been discovered [32], thence the decline of ruminal butyrate in the CON of this trial [15] might partially result from the enrichment of *unclassified_g_Lachnospiraceae_UCG-006*. Moreover, the enrichment of *unclassified_g_Acetitomaculum* in the CON could also explained the ADG that tended to decrease in the same group [15] to some extent, since the ruminal *Acetitomaculum* spp. has been priorly found negatively interconnected with the ADG of sheep [33].

Qiu et al. [34] discovered that the *Rikenellaceae RC9_gut_group* affliated to the *Rikenellaceae* family was positively correlated to the rumen pH, and the ratio of acetate to propionate, which was further verified by the augment of *Rikenellaceae* and its subordinate taxa (*Rikenellaceae RC9_gut_group*, *uncultured_rumen_bacterium_g_Rikenellaceae_RC9_gut_group*, and *uncultured_bacterium_g_Rikenellaceae_RC9_gut_group*) along with the rises in ruminal pH and the acetate-to-propionate ratio [15] presented in the MVWS of this experiment. Besides, it was also reported that *Rikenellaceae RC9_gut_group* and *Monoglobus* were associated with the rumen epithelial morphology in different manners [35, 36]. Therefore, the exact effects of the simultaneously increments in these two genera observed in the MVWS of this study on the rumen epithelium barrier function and the corresponding translocation of ruminal endotoxin needed to be further elaborated, considering the possibly impaired barrier function of gastro-intestine caused by intensive cooling [37]. The *Christensenellaceae R-7_group* is known as a probiotic butyrate-producer that basically decomposes fiber and protein, and it could improve rumen development and immune function [38, 39]. The enrichment of *unclassified_g_Christensenellaceae_R-7_group* noted might thereby partially result in the increases of ruminal butyric acid, immunoglobulin (Ig) A, and IgG in MVWS [15], yet this assumption necessitates further verification.

The bacterial phylum WPS-2 has been occasionally found at relatively minor proportions in the rumen microbiome, with its role in ruminal ecosystem remaining undefined [40, 41]. In this study, the positive correlations between WPS-2, blood endotoxin, and 3-OXO-C6-HSL were noticeable, suggesting that more researches are warranted to disclose the explicit functions of WPS-2 concerning QS and endotoxin translocation. The suppressive effect of *Anaeroplasma* on the cellulolysis by a few ruminal fungi and bacteria, as well as its stimulative impact on the propionate production have been priorly marked [42]. In this trial, the negative and positive correlation of *Anaeroplasma* to the blood endotoxin and urinary endotoxin excretion were respectively shown, implying that *Anaeroplasma* might participate in the endotoxin elimination through urination. Similarly, *Eubacterium_hallii_group* is considered to contribute to the ruminal butyrate production and rumen development [43], and it was also positively connected to the urinary endotoxin level, along with its affiliated uncultured_rumen_bacterium_g_*Eubacterium_hallii_group*. Moreover, the negative correlation between the relative abundance of *Lachnospiraceae* family in fecal microbiota and serum endotoxin has been previously discovered in hepatitis B virus-related liver cirrhosis patients [44], but it was noticed that the genus *norank_f_Lachnospiraceae* positively correlated to the blood endotoxin in this study. This contradiction could stem from the disparities in host and ambience, or the indeterminacy of *norank_f_Lachnospiraceae*, and requires more investigations to be interpreted. *Mogibacterium* is regarded as a pathogenic anaerobe associated with the injured gut health, which could probably facilitate the endotoxin transfer into peripheral blood [45, 46]. In this trial, the negative association between *uncultured_rumen_bacterium_g_Mogibacterium_group* belonging to *Mogibacterium* and endotoxin discharge by urination was displayed, further suggesting the potential involvement of this species in endotoxemia.

In terms of the QS signaling molecules, the positive connection of Planctomycetota to AI-2 was observed, which was similar to the concurrence of increasing abundance in Planctomycetota and enhancement in QS function discovered in surrounding river sediments of a Cu-polymetallic deposit [47]. Furthermore, it was remarkable that both *Sphaerochaeta* and *Acetitomaculum* were positively related to the AI-2 molecule, and the abundance reductions of their affiliated *uncultured_rumen_bacterium_g_ Sphaerochaeta* and *unclassified_g_Acetitomaculum* were also exhibited in MVWS of this study. The relatively low average proportions (< 0.5%) of these two varying species could to some extent serve as a possible explanation for the unchanged AI-2 concentration between treatments. In the current investigation, the positive interrelationship between AI-2 and the *unclassified_g_Lachnospiraceae_NK3A20_group* was partially supported by previous finding that some ruminal *Lachnospiraceae* species could take part in QS [48]. Conversely, the negative interconnection between AI-2 and *unclassified_g_Prevotella* of this trial was inconsistent with precedent reports that the eminent genera *Prevotella* carries abundant AI-2-related genes and plays a critical role in QS amongst the ruminal microbiota [11, 12], necessitating more researches to clarify this dissimilarity.

As demonstrated by the Tax4Fun2 analysis, QS within the rumen bacterial community was augmented by cooling, being contradictory to the aforementioned unaffectedness of 3-OXO-C6-HSL and AI-2 in rumen fluid. In fact, the production and secretion of QS signaling molecules are more of instant and density-dependent activities during a cascade of reactions of the microbial community to ambient stimulations [49], thence it becomes rather challenging to timely detect the subtle variations of QS molecules within the diverse and complex rumen ecosystem upon environmental stresses or changes in vivo [50]. Besides, the limitations of the metagenomic analysis in interpreting the actual activities of the ruminal microflora could also result in the above-discussed contradiction to some extent [22, 51]. Furthermore, since the bacterial chemotaxis and flagellar assembly can help to boost the nutrient utilization of ruminal bacteria and thereby improve the feed efficiency of the host [52], the upregulation of these two pathways might also lead to the increasing tendency of ADG in the bulls treated with cooling [15]. It was noteworthy that the biosynthesis of lipoarabinomannan (LAM), a primary type of the LPS endotoxin of *Mycobacterium tuberculosis* and other mycobacteria [53], was decreased in the MVWS group, which was supported by the above-described abatement of the ruminal endotoxin in the same treatment.

According to the OPLS-DA plot of this study, a clear separation between the samples of the two treatments was portrayed, indicating that cooling reshaped the metabolomic pattern of the rumen liquid in bulls exposed to HS. The subsequent Spearman analysis showed the negative associations between blood endotoxin and several distinct metabolites, as well as the positive interrelationships between ruminal endotoxin, urinary endotoxin excretion, and a few different metabolites. It was prominent that all of these endotoxin-relevant chemicals were downregulated in the MVWS group, possibly leading to the changes of endotoxin across different biofluids by cooling in this study, and these metabolites could be selected as potential biomarkers for preventing and diagnosing the endotoxemia in ruminants [54]. Moreover, a negative relation between 3-OXO-C6-HSL and gingerol was manifested in this study, which was in agreement with precedent investigations that confirmed the inhibitory effect of gingerol on QS and QS-mediated biofilm formation as a structural analog of AHL [55, 56]. As a consequence, the decrement of gingerol in MVWS might somewhat promote the AHL-regulated QS activity despite the unchanged QS signals, which further supported the result of Tax4Fun2 and the difficulty in accurately detecting QS shifts [49, 50]. The salicylic acid has also been classified as a QS inhibitor which hinders the QS-related and virulent gene expressions of *Pseudomonas aeruginosa* [57, 58], the influences of its reduction thereby would probably be similar to that of gingerol. In contrast, the 4-hydroxybenzoic acid is employed by several bacteria as a QS signal in manipulating physiological functions and virulence factors [59], its negative association with 3-OXO-C6-HSL revealed in this trial needs more researches to be verified. Additionally, the simultaneous correlations of 4-hydroxybenzoic acid and salicylic acid with endotoxin were marked, implying the participation of QS in the endotoxin variations of this experiment. The palmitoylcarnitine and DL-2-aminooctanoic acid that were found to be correlated to AI-2 might could serve as possible regulators targeting the QS within ruminal microbiota.

In the current research, the interconnections between cholic acid and a few bacterial species from the genera of *Lactobacillus*, *Eubacterium*, and *Bifidobacterium* were depicted through the MIMOSA2 analysis. Kurdi et al. [60] observed the accumulations of cholic acid by *Lactobacillus* members at different levels, however in this study, the cholic acid amount was negatively and positively related to the *Lactobacillus mucosae_LM1* and *Lactobacillus amylovorus*, respectively. In comparison, it has been reported that members of *Eubacterium* can convert cholic acid into secondary bile acids through 7a-dehydroxylation [61], which was consistent with the negative interrelationships of *Eubacterium cellulosolvens_6* and *Eubacterium limosum* with cholic acid. It can be hence inferred that the rise of cholic acid in MVWS could result from the interactions within these relevant species.

Zhou et al. [62] confirmed that both 3-hydroxybenzoic acid and 4-hydroxybenzoic acid are essential for the production of ubiquinone by a rice bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*. This finding could explain the downregulation of ubiquinone and other terpenoid-quinone biosynthesis in the MVWS of the present experiment, since 3-hydroxybenzoic acid and 4-hydroxybenzoic acid were both decreased in the ruminal fluid of cooling-treated bulls. As exhibited in the diagram of assembled differential pathways, the phenylalanine, tyrosine, and tryptophan biosynthesis that was also reduced in MVWS closely connected to the ubiquinone and other terpenoid-quinone biosynthesis. Phenylalanine is the substrate for the synthesis of catecholamine, an interkingdom signal between the animal and microflora that has been found to increase in response to HS [63–65], whilst tryptophan can act as the precursor of some bioactive compounds involving inflammation [66]. Therefore, the incline of phenylalanine, tyrosine, and tryptophan biosynthesis could to some extent represent the mitigation of HS in the MVWS treatment, and contribute to the decreases of proinflammatory cytokines in the bulls of this experiment [15]. Zhen et al. [67] observed the downregulation of QS

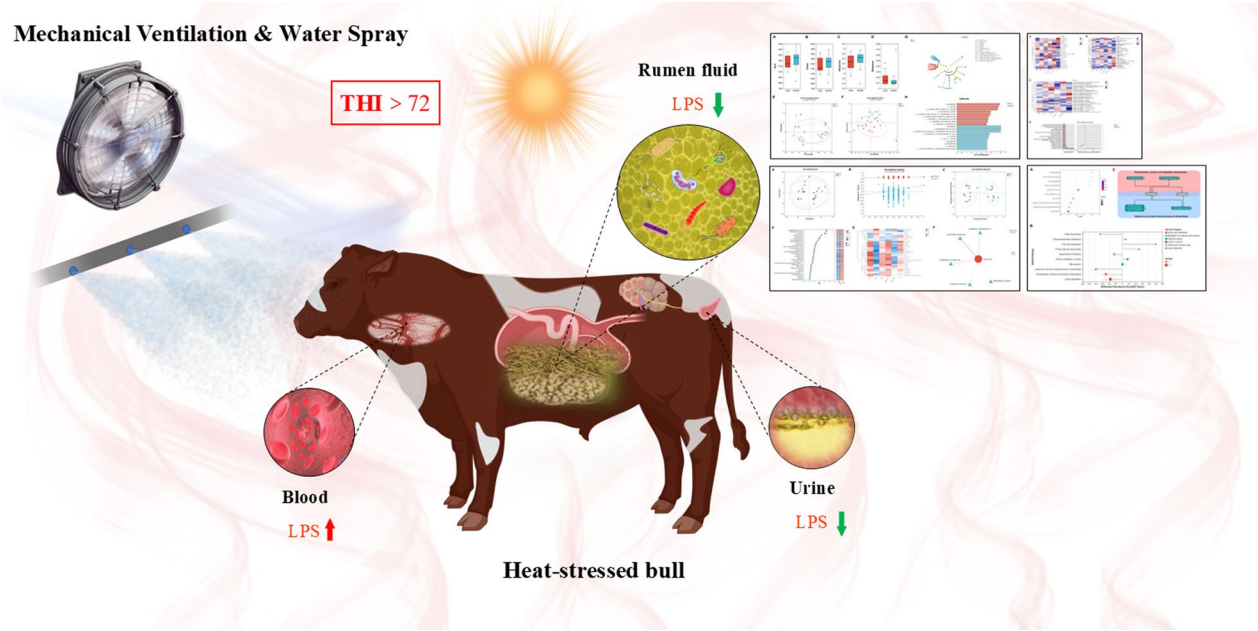

Fig. 6 Overview summary of the present study

together with the upregulation of lysine degradation, and ubiquinone and other terpenoid-quinone biosynthesis in the ileal of hens treated with yeast β-glucan. In the current trial, it was revealed by Tax4Fun2 that QS was upregulated in MVWS, while the metabolomics showed that lysine degradation, and ubiquinone and other terpenoid-quinone biosynthesis were both decreased, suggesting a potentially negative correlations between these two pathways and QS yet requiring deeper verifications. By contrast, the upregulation of bile secretion in MVWS was noted, which could help to boost the digestion and utilization of lipids by the bulls receiving cooling [60].

Conclusion

As was demonstrated by the present investigation, it could be concluded that cooling not only lowered the degree of HS, but also alleviated the harms of HS by redistributing endotoxins across different biofluids in beef bulls. The alterations of endotoxin across different biofluids by cooling involved the manipulations of ruminal microflora and metabolism, as a series of differential bacterial taxa, metabolites, and metabolic pathways were discovered to be associated with endotoxin and QS, despite that the levels of 3-OXO-C6-HSL and AI-2 were unchanged (Fig. 6). Future studies are necessitated to elucidate and testify mechanisms for the mediation of QS in the generation, translocation, and excretion of endotoxin in cattle subjected to HS.

Abbreviations

AI-2	Autoinducer-2
CON	Control
HPLC-FD	Liquid chromatography equipped with a fluorescence detector
HS	Heat stress
KEGG	Kyoto encyclopedia of genes and genomes
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LPS	Lipopolysaccharide
MVWS	Mechanical ventilation and water spray
OPLS-DA	Orthogonal projections to latent structures-discriminant analysis
PCA	Principal components analysis
QS	Quorum sensing
SRA	Sequence read archive
THI	Temperature-humidity index
TMR	Total mixed ration
UHPLC-MS	Ultra-high performance liquid chromatography-mass spectrometry
VIP	Variable importance in projection

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-025-00400-4.

Supplementary material1: Sequencing summary.

Supplementary material2: Identified OTU and taxonomy.

Supplementary material3: Annotated taxa at different levels across samples.

Supplementary material4: LEfSe analysis result.

Supplementary material5: Summary of detected metabolites.

Supplementary material6: Differential metabolites between treatments.

Supplementary material7: Annotated differential pathways and metabolites.

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Author contributions
ZW and WS conceived the ideas and designed the research. ZW, QW, and WS conducted the research. ZW and QW analyzed the data. ZW wrote and revised the manuscript. ZW, WS, FW, JH, LL, ST, and ZT supervised the study. All authors contributed to this study and read and approved the final manuscript.

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Availability of data and materials
All the raw sequences obtained during the sequencing in this study were available at the NCBI database (https://www.ncbi.nlm.nih.gov/bioproject/) with the accession number PRJNA1066970.

Declarations

Ethics approval and consent to participate
All procedures involving animals in this experiment were reviewed and approved by the Animal Care Committee (approval number: 20220626), College of Animal Science and Technology, Hunan Agricultural University, Changsha, China.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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