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Original Research Article

Chromium yeast promotes milk protein synthesis by regulating ruminal microbiota and amino acid metabolites in heat-stressed dairy cows

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ABSTRACT

The intensifying global warming may increase the impact of heat stress on the dairy industry. Our previous study showed that chromium yeast (CY) alleviated the negative effects of heat stress and improved the lactation performance by increasing milk protein content and yield in mid-lactation dairy cows. This study further investigated whether the increased milk protein after CY supplementation results from the promotion of microbial crude protein (MCP) synthesis by regulating rumen microorganisms and amino acid metabolites. Twelve heat-stressed dairy cows were divided into two treatment groups: one with CY supplementation (0.36 mg Cr/kg DM) and the other without CY supplementation. Samples were collected after eight weeks of formal experiment in a hot summer with the mean temperature-humidity index of 79.0 ± 3.13. Dietary CY supplementation did not affect rumen pH, total volatile fatty acid, acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate, but increased ruminal MCP concentration ($P < 0.05$). Simultaneously, the alpha or beta diversity of rumen microbial bacteria were not influenced by CY supplementation. At genus level, supplementation with CY increased the relative abundances of *Olsenella*, *Lachnospiraceae_UCG-002,* and *Shuttleworthia* ($P < 0.05$) and decreased those of *Enterobacter*, *Escherichia-Shigella*, *Oribacterium,* and *Bacteroidetes_BD2-2* ($P < 0.05$). There were 17 up-regulated and 57 down-regulated differential metabolites in the CON and CY groups. The partial least-squares discriminant analysis (PLS-DA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) scores clearly distinguished the two groups. Chromium yeast supplementation reduced the concentrations of D-(+)-proline, DL-glutamic acid, DL-lysine, Gly-l-pro, L-(−)-serine, L-(+)-alanine, and L-(+)-aspartic acid ($P < 0.05$) in the ruminal fluid, which were involved in arginine biosynthesis ($P = 0.029$), glutathione metabolism ($P = 0.047$), lysine degradation ($P = 0.069$), and D-amino acid metabolism ($P = 0.084$). Spearman correlation analysis showed that milk protein content was positively correlated with MCP and negatively correlated with amino acid concentrations in the ruminal fluid ($P < 0.05$). Collectively, CY supplementation promoted the utilization of amino acids by rumen microorganisms to synthesize MCP, thereby increasing milk protein content and yield in heat-stressed dairy cows.

1. Introduction

Milk and dairy products are essential healthy foods for humankind of all ages due to its high quality active protein (Nongonierma and FitzGerald, 2015). With the improvement of people's living standards, the overall consumption demand of milk and dairy products shows a rapid growth trend (Ranjitkar et al., 2020). However, milk production is affected by environmental factors, among which heat stress is often identified as the major

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environmental factors responsible for depressing milk production and quality (Ma et al., 2020; Ranjitkar et al., 2020).

The aggravation of global warming threatens the health and decreases feed intake and lactation performance of dairy cows (Guo et al., 2021). Studies have shown that milk protein content negatively correlates with the temperature and humidity indexes (THI) for indoor and outdoor animals (Hill and Wall, 2015). The contents of milk protein are highest in winter and lowest in summer (Bernabucci et al., 2015), possibly due to the high temperature and humidity in summer (Zhao et al., 2019). Besides, heat stress affects normal rumen metabolism in dairy cows. Zhao et al. (2019) showed that heat stress increases the relative abundance of lactate-producing bacteria but decreases that of acetate-producing bacteria. Tajima et al. (2007) also demonstrated that heat stress significantly impacts the ruminal microbiota composition of Holstein heifers.

Chromium (Cr) is an essential mineral participating in glucose metabolism in the body (Lashkari et al., 2018). Chromium supplementation has been considered as a promising agent for resisting the adverse effects of heat stress and improving livestock health and production (Bin-Jumah et al., 2020). A previous study showed that chromium yeast (CY), a kind of organic form of Cr, alleviates the negative effects of heat stress and improves lactation performance by increasing the milk protein content and yield in mid-lactation dairy cows (Wo et al., 2023), but the underlying mechanisms remain unknown. Wo et al. (2023) revealed that CY influences the endocrine function and glucose metabolism of heat-stressed dairy cows and contributes to the reduction in rectal temperature. Considering that lactation is a complex process during which rumen microbiota also play a vital role, we hypothesized that the increased milk protein synthesis might be due to the variation of ruminal microbiota and metabolites after CY supplementation. To test this hypothesis, we investigated the influence of CY supplementation on the changes in ruminal microbiota and metabolomics. The MCP concentrations in the rumen of heat-stressed cows was also determined and then correlation analysis was conducted to calculate the Spearman correlation coefficient between microbes, metabolites, MCP concentrations, and milk proteins.

2. Materials and methods

2.1. Animal ethics statement

The Ethics Committee of the Chinese Academy of Agricultural Sciences (ethics code permit: IAS 2019-8) approved all the procedures in this study. The dairy cows were cared for following the standards established by the Institute of Animal Science, Chinese Academy of Agricultural Sciences (Beijing, China).

2.2. Animals, diets, and experimental design

Twelve mid-lactation Holstein dairy cows (days in milk 125 ± 8 days, milk yield 24.6 ± 1.5 kg/day, parity 2 or 3) were divided into two groups: the control (CON, cows received basic total mixed ration, $n = 6$) and CY (cows received total mixed ration [TMR] supplemented with CY at 0.36 mg Cr/kg DM, $n = 6$) groups. The chromium yeast containing 996 mg/kg Cr was obtained from China Angel Yeast Co., Ltd. (Yichang City, Hubei Province, China). All the dairy cows were exposed to heat-stress conditions for 10 weeks including two weeks pre-experiment (THI = 73.4 ± 1.27) and eight weeks of the formal experiment (THI = 79.0 ± 3.13). All cows were fed the same TMR without CY supplementation. Table 1 shows the composition and nutritional content of the TMR used. The nutrient levels met or exceeded the National Research Council (2001) recommendations for dairy cows. The concentration of Cr in the CY and TMR was determined using inductively coupled plasma mass spectrometry/mass generation (ICP-MS/MS, Agilent 8800, Agilent Technologies, Santa Clara, CA, USA) according to the National Standard for Food Safety of China (GB 5009.268-2016) with some modifications (Shan et al., 2020; Wo et al., 2023). Briefly, CY (0.1 g) or TMR (1.0 g) was digested after addition of nitric acid and perchloric acids in an electrically heated digestion block, and then deionized water was added to 25 mL. Thereafter, inductively coupled plasma mass spectrometry/vapor generation was used to analyze the concentration of Cr. The background Cr concentration in the TMR was 0.09 mg Cr/kg DM. Standard procedures of the Association of Official Analytical Chemists were used to determine dry matter (AOAC, 2005; method 930.15) and crude protein (AOAC, 2000; method 976.05). The content of neutral detergent fiber (NDF) and acid detergent fiber (ADF) was determined as described by Van Soest et al. (1991). Calcium (AOAC, 1990; method 985.35) and phosphorus (AOAC, 1990; method 986.24) contents were analyzed by atomic absorption spectroscopy and spectrophotometry, respectively. Net energy for lactation was a calculated value according to NRC (2001). All the cows were milked three times daily. Additional details regarding the feeding milking and management of the dairy cows have been published previously (Wo et al., 2023).

Table 1
Ingredients and nutrient levels of the basal diet (% of DM).

Item	Content
Ingredients	
Corn silage	27.70
Ground corn	14.80
Ryegrass[1]	12.10
Soybean meal	9.10
Oats	7.70
Flaked corn	7.00
Sugar beet pulp	3.80
Whole cottonseed	3.10
Syrup	2.90
Distiller's dried grains	2.20
Alfalfa hay	2.10
Yeast culture[2]	1.20
Chinensis	1.10
Fat powder[3]	1.10
Limestone	1.00
$NaHCO_3$	0.70
$CaHPO_4$	0.40
NaCl	0.40
K_2CO_3	0.20
MgO	0.10
Premix[4]	1.30
Total	100.00
Nutrient levels[5]	
CP	15.90
NDF	26.09
ADF	13.75
Ca	0.83
P	0.47
Cr, mg/kg	0.09
NE_L[6], MJ/kg	6.88

[1] Ryegrass green chopped.
[2] Diamond V XP yeast culture supplement (Diamond V, USA).
[3] A saturated free fatty acid supplement (Bergafat, Berg + Schmidt, Germany).
[4] The premix provided the following per kg of the diet: vitamin A 200,000 IU, vitamin D 40,000 IU, vitamin E 5,000 IU, Mg 95,200 mg, Zn 1,000 mg, Co 50 mg.
[5] Analyzed value.
[6] Net energy for lactation was a calculated value according to NRC (2001).

2.3. Sampling and analysis

Cows were milked at 05:30, 12:30 and 19:30 every day and daily milk production was recorded. Milk samples were collected weekly

from each cow over three consecutive milkings, mixed at a ratio of 4:3:3, with preservatives (Bronopol Tablet, D&F Control System, San Ranmon Inc., Dublin, ON, Canada) added, and stored at 4 °C (Wo et al., 2023). Milk fat, protein, lactose, solid not fat and total solid were measured using infrared analysis (FOSS MilkoScan 2000; FOSS Food Technology Corp., Eden Prairie, MN, USA). On day 56, ruminal fluid samples were collected 2 h after morning feeding. Rumen fluid samples were collected from each cow using the stomach tube type ruminal fluid sampler (Wuhan kolibo animal science and technology Co., Ltd., China). The first 100 mL of ruminal fluid per cow was discarded to minimize contamination with saliva. Then, a total of 200 mL of ruminal fluid sample was collected from each cow, and the pH was measured. The samples were filtered through four layers of cheesecloth and divided into three parts for subsequent analysis of volatile fatty acids (VFA), ruminal bacteria, and metabolites, respectively. The VFA (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid) concentrations in ruminal fluids were determined using gas chromatography (7890A, Agilent, CA, USA) as described previously (Sun et al., 2016). Rumen MCP concentration was measured by urinary purine derivative excretion using a previously reported method (Chen and Gomes, 1992).

2.4. Microbial diversity analysis of ruminal fluid

Total microbial DNA from ruminal fluid was extracted using the M5635-02 kit (Omega, Bio-Tek, GA, USA), following the manufacturer's strict instructions. Nanodrop NC2000 (Thermo Fisher Scientific, MA, USA) was used to quantify the DNA concentration and purity, and the DNA quality was assessed using 1.2% agarose gel electrophoresis. Passeno Biotechnology Co., Ltd. (Shanghai, China) provided the primers and the Pfu high-fidelity DNA polymerase (Beijing Quanshijin Biotechnology Co., Ltd., Beijing, China) was used for PCR amplification in the ABI2720 PCR amplifier (Applied Biosystems, MA, USA). The bacterial 16S rRNA gene V3–V4 region was amplified by PCR using the forward primer (338F primers 5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer (806R primers 5′-GGACTACHVGGGTWTCTAAT-3′). The PCR program was 98 °C for 2 min, followed by 30 cycles of 98 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension of 5 min at 72 °C. The PCR reaction mixture: 4 μL of 5 × PrimeSTAR buffer, 2μL of 2.5 mmol/L dNTP, 0.8 μL of primer (5 mmol/L), 0.4μL of PrimeSTAR HS DNA polymerase (TaKaRa, Dalian, China), and 20 ng of template DNA were added to fill the volume to 20μL. Amplicons were purified using an AxyPrep DNA gel extraction kit according to the manufacturer's instructions (Axygen Biosciences, Union City, CA, USA) and sequenced on the illumina novaseq6000 sequencing platform. The sequences were submitted to GenBank, with the accession number PRJNA1098615.

Sequence quality control followed the dada2 plugin in Quantitative Insights Into Microbial Ecology (QIIME 2) for denoising, ligation, and chimera removal to obtain amplicon sequence variants (ASV) using the classify-sklearn algorithm. Raw sequence data were demultiplexed using the demux plugin, followed by primer trimming with the cutadapt plugin. The sequences were then quality filtered, denoised, merged and made chimera free using the DADA2 plugin. Non-singleton ASV were aligned with MAFFT (Katoh et al., 2002) and a phylogeny was constructed with FastTree2 (Price et al., 2009). The ASV data were normalized using total sum-scaling (TSS) normalization to obtain the relative abundances of microbial taxonomic ranks, addressing uneven sequencing depth. Alpha-diversity and beta diversity metrics were then estimated using the diversity plugin, with samples rarefied to 80,948 sequences per sample. Beta-diversity was visualized using bray_curtis (Bray and Curtis, 1957) principal coordinates analysis (PCoA) (Ramette, 2007). Finally, linear discriminant analysis (LDA) combined with effect size measurements (LEfSe) (Segata et al., 2011) and random forests algorithm (Breiman, 2001) was used to identify biomarkers. Microbial functions were predicted by phylogenetic analysis of communities through reconstruction of unobserved states (PICRUSt2) based on MetaCyc databases (Caspi et al., 2016; Douglas et al., 2020). Differential analysis of the MetaCyc metabolic pathways was performed using the metagenomeSeq package in R.

2.5. Metabolomics analysis of ruminal fluid

Metabolomic analysis of the Ruminal fluid samples were performed by liquid chromatography mass spectrometry (LC-MS)/MS analysis. Briefly, 100 μL of each sample was transferred to a 1.5-mL Eppendorf tube and mixed with 1 mL of pre-chilled chloroform:methanol:water (1:2:1). The mixture was vortexed and allowed to stand at 4 °C for 2 h. Next, the supernatant was obtained by centrifugation at 13,000 × *g* for 15 min and was filtered through a 0.22-μm membrane for LC-MS analysis. The samples were separated using a DIONEX UltiMate 3000 UPLC (Dionex, CA, USA) using a C18 column followed by UPLC separation. The injection volume was 3 μL, the column temperature was 45 °C, and the flow rate was 0.35 mL/min. Chromatographic mobile phase A: 0.1% formic acid water, B: acetonitrile (0.1% formic acid). Quality control samples were inserted into the sample cohort to monitor and evaluate the stability of the system and the reliability of the experimental data. They were analyzed by a Q-Exactive mass spectrometer (Thermo

Table 2
Effects of chromium yeast on rumen fermentation in heat-stressed dairy cows.[1]

Item	Treatments[2]		*P*-value
	CON	CY	
MCP, g/d	1057.03 ± 63.272	1249.36 ± 37.787	0.026
pH	6.77 ± 0.056	6.67 ± 0.071	0.296
Total volatile fatty acid, mmol/L	151.85 ± 12.122	159.69 ± 9.437	0.621
Acetate, mmol/L	96.78 ± 8.113	99.35 ± 4.606	0.789
Propionate, mmol/L	30.47 ± 2.321	34.93 ± 3.611	0.321
Isobutyrate, mmol/L	1.28 ± 0.065	1.39 ± 0.099	0.376
Butyrate, mmol/L	18.68 ± 2.106	19.54 ± 1.646	0.755
Isovalerate, mmol/L	2.19 ± 0.174	2.07 ± 0.134	0.607
Valerate, mmol/L	2.34 ± 0.209	2.52 ± 0.133	0.465
Acetate/propionate	3.19 ± 0.180	2.96 ± 0.237	0.468

MCP = microbial crude protein.
[1] Data are means ± SEM.
[2] CON, control group ($n = 6$, without chromium yeast supplementation); CY, chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM).

Fisher, CA, USA). Quality control samples with equal volume of all samples were prepared to optimize the LC-MS/MS system and evaluate its stability. The raw data for peak alignment was subjected to retention time correction, and the peak areas were corrected using Compound Discoverer 3.0 (Thermo Fisher Scientific, MA, USA). Accurate mass matching (<25 ppm) and secondary spectrum matching were performed in the MZcloud database (https://www.mzcloud.org/) to identify ruminal fluid metabolites. Metabolites with >50% missing values in the raw data were discarded. Unsupervised principal component analysis (PCA), PLS-DA, and OPLS-DA were used to determine the differences in sample metabolite composition between the CON and CY groups. The significantly different metabolites were annotated in the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) to enrich the metabolic pathways.

2.6. Statistical analysis

All data were analyzed using SAS version 9.4 (SAS Institute Inc., NC, USA). The ruminal fermentation parameters (MCP, pH, and VFA) were analyzed by Student's *t*-test. The α diversity (Chao1, Observed_species, Shannon, and Simpson indexes) and the relative abundances of ruminal bacterial phyla and genera were assessed using the non-parametric Wilcoxon test. The results are shown as

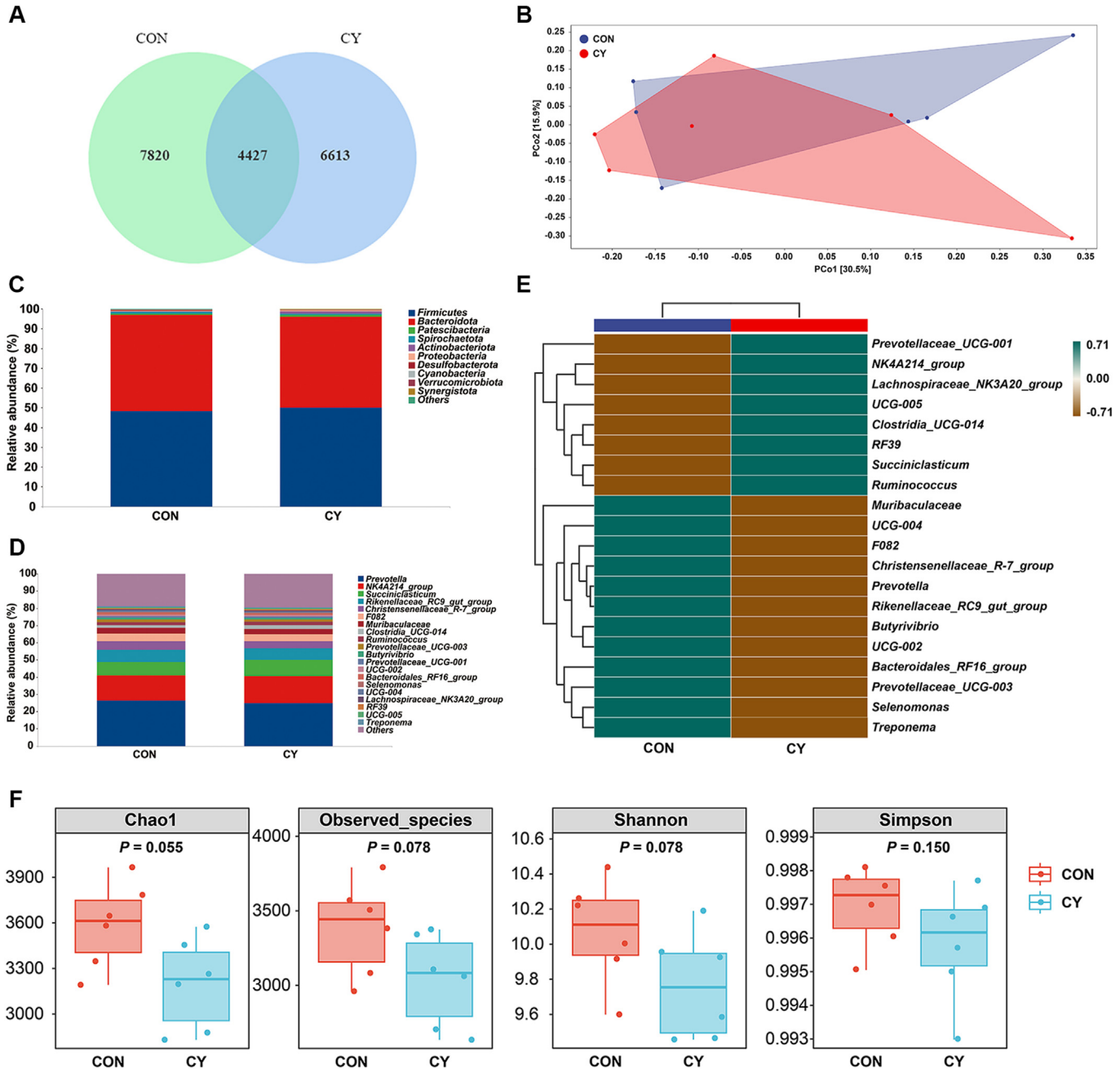

Fig. 1. Effects of chromium yeast supplementation on the composition and differences in ruminal microbial community in heat-stressed dairy cows. (A) Venn diagram of amplicon sequence variant (ASV), (B) PLS-DA plots, (C) phylum, and (D) genus-level relative abundances, (E) heatmap of species composition at the genus-level relative abundances, and (F) Chao1, Observed_species, Shannon and Simpson indexes. CON = control group (n = 6, without chromium yeast supplementation); CY = chromium yeast group (n = 6, received additional chromium yeast at 0.36 mg Cr/kg DM).

least squares of means and standard errors of the mean. Differences with $P < 0.05$ were considered statistically significant, and the statistical trends were $0.05 < P < 0.10$. The relationships among the rumen microbes, metabolites, and production performances were analyzed using Spearman's rank correlation coefficients, and the screening threshold was $R = 0.6$.

3. Results

3.1. Effects of CY on the ruminal fermentation and MCP of heat-stressed dairy cows

Chromium yeast supplementation did not affect rumen pH, total volatile fatty acid, acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate ($P > 0.05$), but significantly increased the content of MCP in the rumen ($P < 0.05$, Table 2).

3.2. Effects of CY on the ruminal microbial diversity of heat-stressed dairy cows

A total of 1,705,724 raw reads were obtained across the CON and CY groups, and 1,601,559 valid tags were generated after screening, accounting for 93.9% of the raw reads. A Venn diagram of ASV showed that the common ASV between CON and CY groups was 4427 with that of CON and CY groups was 7820, and 6613, respectively (Fig. 1A). However, the PCoA plot showed non-significant differences between the two groups (Fig. 1B). Fig. 1C&D showed the bacterial species composition of ruminal

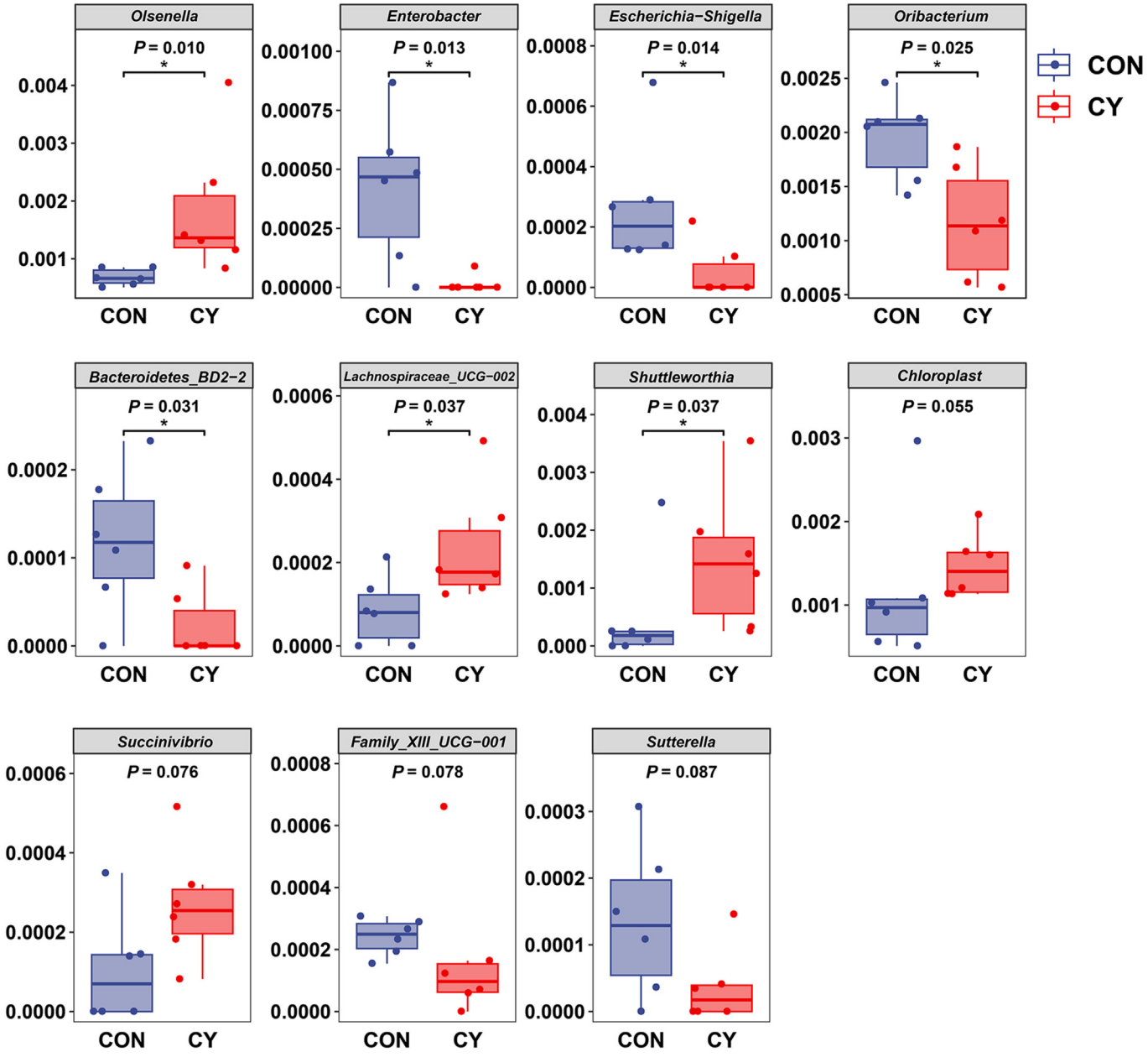

Fig. 2. Differences between the relative abundances of bacterial genera in ruminal fluids of heat-stressed dairy cows. The abundances of specific genera were compared among the CON and CY groups: (A) *Olsenella*. (B) *Enterobacter*. (C) *Escherichia-Shigella*. (D) *Oribacterium*. (E) *Bacteroidetes_BD2-2*. (F) *Lachnospiraceae_UCG-002*. (G) *Shuttleworthia*. (H) *Chloroplast*. (I) *Succinivibrio*. (J) *Family_XIII_UCG-001*. (K) *Sutterella*. * Significance with $P < 0.05$. CON = control group ($n = 6$, without chromium yeast supplementation). CY = chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM).

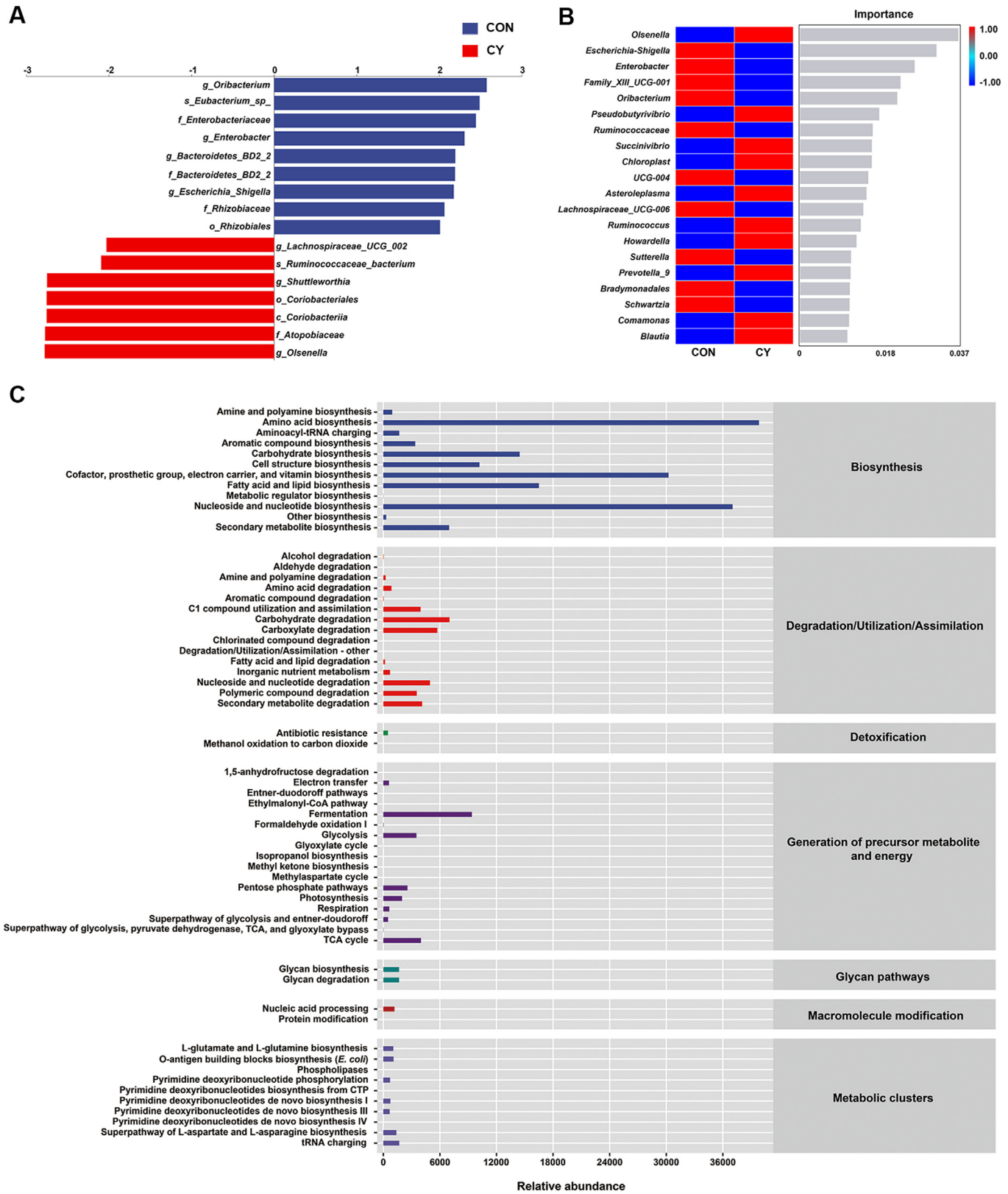

Fig. 3. Effects of dietary CY supplementation on biomarkers and metabolic pathways in the rumen of heat-stressed dairy cows. (A) LDA effect size analysis. (B) Random forest analysis. (C) MetaCyc predicted microbial metabolic functions, CON = control group ($n = 6$, without chromium yeast supplementation). CY = chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM). LDA = linear discriminant analysis.

fluid samples at the phylum (top 10) and genus (top 20) levels. At the phylum level, Firmicutes was the dominant phylum, followed by Bacteroidota, Patescibacteria, and Spirochaetota (Fig. 1C). At the genus level, *Prevotella* was the dominant genus (Fig. 1D). Moreover, we used the data of the top 20 genera of the abundance to draw Fig. 1E. The Chao1, Observed_species and Shannon indexes in the CY group tended to decrease ($0.05 < P < 0.10$, Fig. 1F).

At the genus level, *Olsenella*, *Lachnospiraceae_UCG-002,* and *Shuttleworthia* were significantly more abundant in CY group than in the CON group ($P < 0.05$, Fig. 2A, F and G). However, the CON group had significantly higher relative abundances of *Enterobacter*, *Escherichia-Shigella*, *Oribacterium*, and *Bacteroidetes_BD2-2* than the CY group ($P < 0.05$, Fig. 2B–E). Addition of CY tended to increase the relative abundances of *Chloroplast* and *Succinivibrio* ($0.05 < P < 0.10$, Fig. 2H and I) and decrease those of *Family_XIII_UCG-001* and *Sutterella* ($0.05 < P < 0.10$, Fig. 2J and K).

As shown in Fig. 3A, four microbiota biomarkers, *Bacteroidetes_BD2_2*, *Escherichia_Shigella*, *Enterobacter* and *Oribacterium,* were enriched in the CON group, while three other indicators, *Lachnospiraceae_UCG_002*, *Shuttleworthia*, and *Olsenella* were enriched in the CY group. Furthermore, the biomarkers identified in the random forest algorithm included *Olsenella*, *Escherichia-Shigella*, *Enterobacter*, *Family_XIII_UCG-001*, and *Oribacterium* (Fig. 3B).

MetaCyc predicted microbial metabolic functions were related to biosynthesis, degradation/utilization/assimilation, detoxification, generation of precursor metabolite and energy, glycan pathways, macromolecule modification, and metabolic clusters. The enriched pathways function in amino acid biosynthesis, nucleoside and nucleotide biosynthesis, cofactor, prosthetic group, electron carrier, vitamin biosynthesis, fatty acid and lipid biosynthesis, and carbohydrate biosynthesis (Fig. 3C). Compared with CON group, the microbial metabolic pathways of Chondroitin sulfate degradation I, Chorismate biosynthesis II, Enterobactin biosynthesis, Superpathway of L-threonine metabolism, Norspermidine biosynthesis, Superpathway of chorismate metabolism, Palmitate biosynthesis II and 4-hydroxyphenylacetate degradation were significantly down-regulated in CY group, while 3-phenylpropanoate and 3-(3-hydroxyphenyl) propanoate degradation, 3-phenylpropanoate and 3-(3-hydroxyphenyl) propanoate degradation to 2-oxopent-4-enoate and Cinnamate and 3-hydroxycinnamate degradation to 2-oxopent-4-enoate were significantly up-regulated (Table 3).

3.3. Effects of CY on the ruminal metabolites of heat-stressed dairy cows

Unsupervised PCA, PLS-DA, and OPLS-DA characterized the differences in metabolic profiles between the CON and CY groups (Fig. 4A and B, and C). The PLS-DA and OPLS-DA score plots showed significantly different metabolite compositions between the two groups (Fig. 4B and C). Treatment with CY differentially expressed 74 ruminal metabolites (17 significantly up-regulated and 57 significantly down-regulated) (Fig. 4D and E, Table S1, $P < 0.05$). Nonetheless, CY supplementation significantly down-regulated ruminal D-(+)-proline, DL-glutamic acid, DL-lysine, Gly-l-pro, L-(−)-serine, L-(+)-alanine, and L-(+)-aspartic acid concentrations (Fig. 5A, $P < 0.05$). The KEGG analysis of the metabolites showed that they enriched in eight metabolic pathways: arginine biosynthesis ($P = 0.029$), glutathione metabolism ($P = 0.047$), lysine degradation ($P = 0.069$), D-amino acid metabolism ($P = 0.084$), drug metabolism-cytochrome P450 ($P = 0.105$), biosynthesis of amino acids ($P = 0.152$), 2-oxocarboxylic acid metabolism ($P = 0.169$), and metabolic pathways ($P = 0.684$, Fig. 5B).

3.4. Correlation between ruminal metabolites and lactation performance of heat-stressed dairy cows

Figure 5C shows the correlations between ruminal metabolites and lactation performance of heat-stressed dairy cows. Microbial crude protein concentration was negatively correlated with the concentration of DL-glutamic acid, L-(−)-serine, L-(+)-alanine, and L-(+)-aspartic acid ($P < 0.01$).The milk yield and milk proteins were negatively correlated with DL-glutamic acid, DL-lysine, Gly-l-pro, L-(−)-serine, L-(+)-alanine, and L-(+)-aspartic acid ($P < 0.05$). The following parameters were negatively correlated: milk fat with L-(+)-alanine ($P < 0.05$), lactose with DL-lysine and L-(−)-serine ($P < 0.05$), solid not fat with D-(+)-proline, DL-glutamic acid, L-(−)-serine, and L-(+)-aspartic acid ($P < 0.05$), and total solid with D-(+)-proline, DL-glutamic acid, L-(+)-alanine, and L-(+)-aspartic acid ($P < 0.05$).

3.5. Correlation between the ruminal microbiota (genera) and the lactation performance of heat-stressed dairy cows

Figure 6A shows the correlation between the relative abundances of microbiota with the lactation performance. The milk protein was positively correlated with the ruminal MCP concentration ($P < 0.001$), and negatively correlated with the relative abundance of *Sutterlla* ($P < 0.05$). Milk fat content was negatively correlated with the relative abundance of *Family_XIII_UCG-001* ($P < 0.01$). Milk lactose content was positively correlated with the relative abundances of *Olsenella* and *Lachnospiraceae_UCG-002* ($P < 0.05$), and negatively correlated with those of *Enterobacter*, *Escherichia-Shigella*, and *Oribacterium* ($P < 0.05$). Milk solid not fat was positively correlated with the relative abundances of

Table 3
Differential microbial metabolic pathways in CON group and CY group.[1]

Pathway	LogFC	SE	*P*-value
Chondroitin sulfate degradation I	−1.77	0.60	0.003
Chorismate biosynthesis II	−0.29	0.11	0.006
Enterobactin biosynthesis	−1.48	0.56	0.009
Superpathway of L-threonine metabolism	−1.70	0.67	0.012
Norspermidine biosynthesis	−1.56	0.66	0.018
Superpathway of chorismate metabolism	−0.93	0.43	0.030
Palmitate biosynthesis II	−0.29	0.14	0.033
4-Hydroxyphenylacetate degradation	−1.02	0.50	0.043
3-Phenylpropanoate and 3-(3-hydroxyphenyl) propanoate degradation	1.70	0.84	0.043
3-Phenylpropanoate and 3-(3-hydroxyphenyl) propanoate degradation to 2-oxopent-4-enoate	1.75	0.88	0.046
Cinnamate and 3-hydroxycinnamate degradation to 2-oxopent-4-enoate	1.75	0.88	0.046

FC = fold change; SE = standard error.
[1] CON, control group ($n = 6$, without chromium yeast supplementation); CY, chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM).

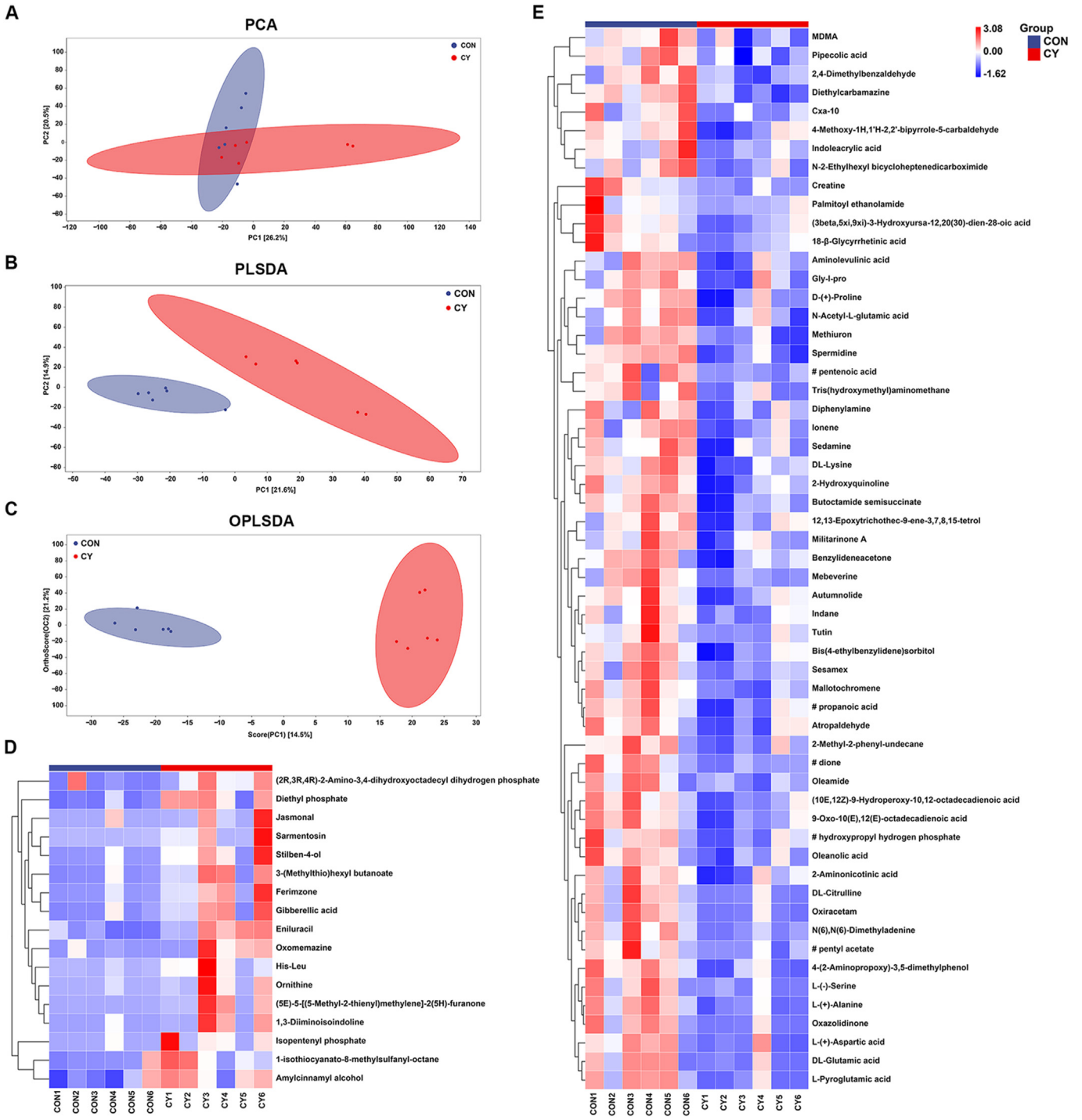

Fig. 4. Effects of dietary chromium yeast supplementation on rumen metabolites. The rumen metabolism of heat-stressed dairy cows in the CON and CY groups were as follows: (A) The principal component analysis (PCA) plots [R^2X (cum) = 0.617]. (B) The partial least-squares discriminant analysis (PLS-DA) plots [R^2X = 0.365,R^2Y = 0.95, Q^2 = 0.346]. (C) The orthogonal partial least-squares discriminant analysis (OPLS-DA) score plots [R^2X = 0.514, R^2Y = 0.984, Q^2 = 0.491]. (D&E) Hierarchical clustering of the differential metabolites ($P < 0.05$, variable importance for the projection [VIP] > 1.0). CON = control group ($n = 6$, without chromium yeast supplementation). CY = chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM).

Olsenella, *Lachnospiraceae_UCG-002* and *Chloroplast*, and MCP concentration ($P < 0.05$), and negatively correlated with those of *Enterobacter* and *Escherichia-Shigell* ($P < 0.05$). Milk total solid was positively correlated with ruminal MCP concentration ($P < 0.05$), and negatively correlated with the relative abundance of *Enterobacter* ($P < 0.05$).

3.6. Correlation between ruminal metabolites and ruminal microbiota (genera) of heat-stressed dairy cows

Interaction heatmaps reflected the relationships between ruminal microbiota and metabolites of the CON and CY groups (Fig. 6B). D-(+)-Proline was positively correlated with the relative

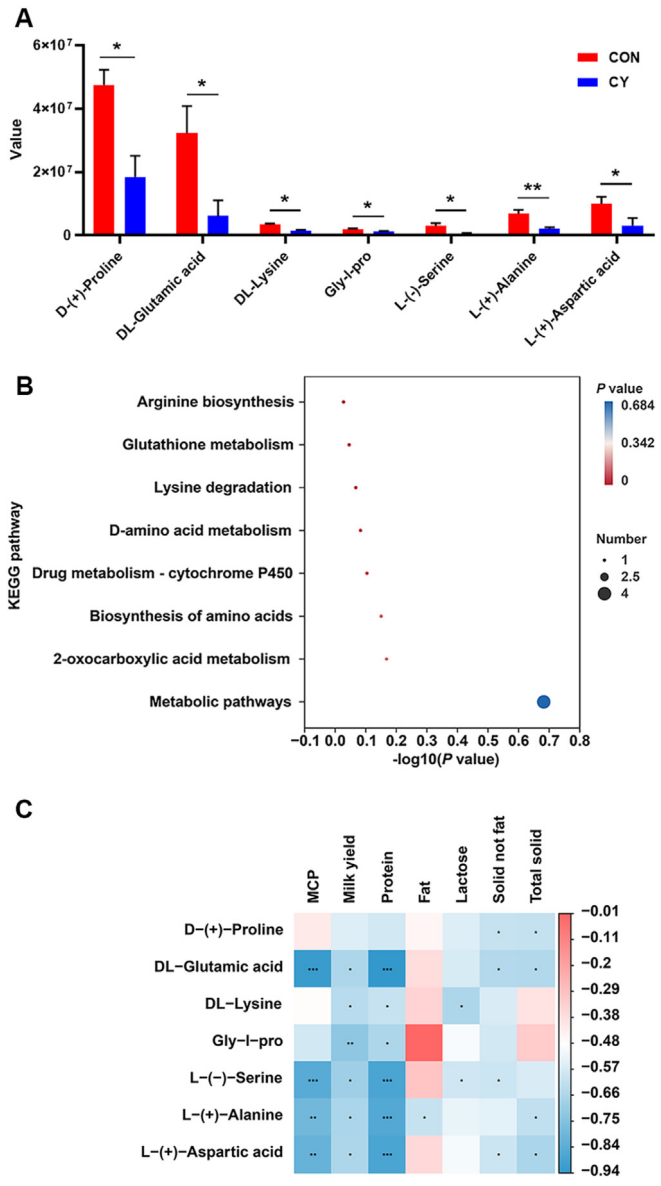

Fig. 5. Correlation of ruminal amino acids with ruminal microbial crude protein (MCP) centration, milk yield, and milk composition of heat-stressed dairy cows in the two groups. (A) Abundance of amino acid. (B) Enrichment analysis of differential metabolites in rumen. (C) Correlation of differential amino acids with MCP, milk yield, and milk composition. * Significance with $P < 0.05$, ** Significance with $P < 0.01$, *** Significance with $P < 0.001$. CON = control group ($n = 6$, without chromium yeast supplementation). CY = chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM).

abundances of *Enterobacter* and *Escherichia-Shigella* ($P < 0.05$) and negatively correlated with *Chloroplast* ($P < 0.05$). DL-Lysine was positively correlated with the relative abundances of *Escherichia-Shigella* and *Bacteroidetes_BD2-2* ($P < 0.05$) but negatively correlated with that of *Shuttleworthia* ($P < 0.01$). In the both groups, the relative abundance of *Sutterella* was positively correlated with DL-glutamic acid, DL-lysine, L-(−)-serine, and L-(+)-alanine ($P < 0.05$). Gly-l-pro was negatively correlated with the relative abundance of *Chloroplast* ($P < 0.05$).

4. Discussion

Cows regulate their body temperature to maintain normal body metabolism. Heat-stressed dairy cows achieve heat balance mainly by speeding up heat dissipation. Our previous study has showed that CY alleviates the negative effects of heat stress in dairy cows by reducing the rectal temperature and increasing plasma nicotinamide concentration for heat dissipation, thus contributed to the promotion of lactation performance by increasing milk and protein yield, but not significantly affecting dry matter intake (Wo et al., 2023).

It is known that protein metabolism is related to glucose metabolism. In the body, protein synthesis requires glycogen as an energy source (Cruz-Pineda et al., 2022). Wo et al. (2023) demonstrated that CY supplementation reduced serum insulin concentration and increased serum glucose concentration in heat-stressed dairy cows, which might promote more energy for the synthesis of milk protein. On the other hand, Cr is an active component in glucose tolerance factor and potentiates the action of insulin, which can also prevent proteolysis and promote protein synthesis. Although Wo et al. (2023) showed that the serum insulin concentrations were reduced by CY supplementation, the insulin sensitivity was not influenced between the dairy cows from the two groups. However, how Cr is involved in milk protein synthesis still needs further investigation.

Considering the precursors of milk protein originate from nutrients absorbed by the gastrointestinal tract, here, we further explored the mechanism of CY in promoting milk protein synthesis in heat-stressed dairy cows by investigating the variation of rumen MCP concentration, ruminal microbiota, and rumen metabolites. Cow rumen is a relatively stable anaerobic environment (Matthews et al., 2019) and the relative stability of the ruminal pH and VFA are crucial for maintaining the stability of the ruminal environment. In the present study, CY supplementation did not affect ruminal pH or VFA. Similar results have been obtained by Zhao et al. (2023) who found that feeding chromium propionate to postpartum heat-stressed Holstein dairy cows had no significant effect on ruminal isobutyrate, valerate, and isovalerate concentrations.

The complex microbial communities in the ruminal ecosystem play a vital role in the health of the gastrointestinal tract (Zeineldin et al., 2018). Previous studies have shown that *Olsenella* is a beneficial actinomycete bacterium that forms part of the normal bacterial flora in the intestinal tract (Kong et al., 2019; Zhang et al., 2014). *Shuttleworthia*, a promising target for regulating rumen function, is positively correlated with fibre digestion (Zhang et al., 2017) and MCP production (Hao et al., 2021). *Lachnospiraceae_UCG-002* belongs to butyrate-producing bacteria and may be involved in antioxidant and anti-inflammatory effects (Chen et al., 2024; Kleuskens et al., 2022; Li et al., 2022; Mancabelli et al., 2017). However, *Escherichia-shigella* is associated with inflammatory bowel disease and intestinal infection (Ferdous et al., 2024; Wang et al., 2024; Ye et al., 2024). *Enterobacter* is also a common pathogen with urinary tract infection (Al-Shahrani and Belali, 2024). In the present study, supplementation with CY increased the relative abundances of *Olsenella*, *Lachnospiraceae_UCG-002*, and *Shuttleworthia*, but declined those of *Sutterella*, *Enterobacter* and *Escherichia-shigella*, suggesting that CY supplementation might help improve the digestive function and reduce pathogenic bacteria in the rumen of heat-stressed dairy cows, which still needs further investigation.

The MCP in the rumen is an ideal protein source for ruminants, which is closely related to protein metabolism. It provides 50% of the small intestine absorbable protein for ruminants, and its production is affected by sufficient adequate adenosine triphosphate and nitrogen originated from non-protein and protein nitrogen sources (Hao et al., 2021; Shi et al., 2023). Microbial crude protein contains more than half of the metabolizable proteins in dairy cows and it has essential amino acid composition similar to milk (Schwab and Broderick, 2017). Studies have shown that the increase of milk protein yield may be related to the increase of MCP (Chen et al., 2024), because MCP is considered to be synthesized by rumen

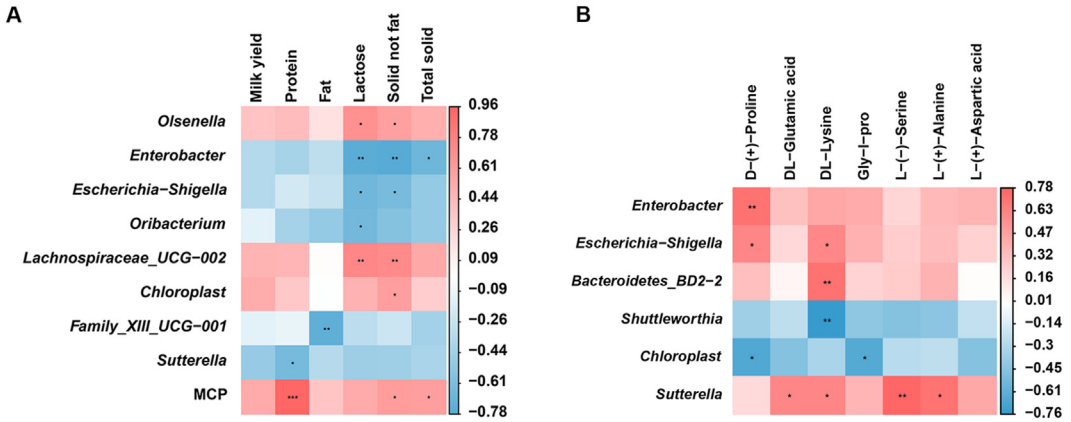

Fig. 6. Correlation between genus-level relative abundances of rumen microbial bacteria with milk yield, milk composition, and amino acids. (A) Correlation between the genus-level relative abundances with milk yield and composition. (B) Correlation between genus-level relative abundances and amino acids. * Significance with $P < 0.05$, ** Significance with $P < 0.01$, *** Significance with $P < 0.001$.

microorganisms with NH_3–N, peptide and amino acids (Sun et al., 2016). Previous evidence has shown that rumen levels of free amino acid were higher in cows with <3.4% milk protein concentration than in those with high milk protein (≥3.4% milk protein concentration) (Wang et al., 2022). Generally, the correlation of the ruminal amino acid contents with ruminal MCP concentration, milk yield, and milk composition of heat-stressed dairy cows in the two groups showed that the amino acid contents in the rumen was negatively correlated with either ruminal MCP concentration or lactation performance of heat-stressed dairy cows. Furthermore, ruminal MCP concentration was positively correlated with milk protein yield. In the present study, CY supplementation significantly elevated MCP production but decreased the contents of D-(+)-proline, DL-glutamic acid, DL-lysine, Gly-l-pro, L-(−)-serine, L-(+)-Alanine, and L-(+)-aspartic acid in the rumen, indicating that more amino acids had been used to synthesize MCP, which finally increased milk protein content and yield.

5. Conclusions

The present study provides important evidence for the mechanism of CY supplementation to increase milk protein production by regulating rumen microbiota and metabolites, reducing the relative abundances of potentially pathogenic bacteria, thus promoting MCP synthesis via fully utilizing ruminal free amino acids in the rumen of heat-stressed dairy cows. These findings suggested that adding an appropriate amount of CY to the diet is a feasible strategy for not only alleviating the negative effects but suppressing the decreased lactation performance induced by heat stress in dairy cows during hot summers.

CRediT authorship contribution statement

Qiang Shan: Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Fengtao Ma:** Writing – review & editing, Methodology, Investigation. **Qi Huang:** Writing – review & editing, Methodology. **Yeqianli Wo:** Writing – review & editing, Methodology. **Peng Sun:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Availability of data and materials

The 16S rRNA sequencing data for all samples have been deposited into the NCBI Sequence Read Archive database (project number, PRJNA1098615).

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.11.003.

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