Dietary Supplementation With Creatine Pyruvate Alter Rumen Microbiota Protein Function in Heat-Stressed Beef Cattle

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Abstract

Background: Creatine pyruvate (CrPyr) is a new multifunctional nutrient that can provide both pyruvate and creatine. It has been shown to relieve the heat stress of beef cattle by improving antioxidant activity and rumen microbial protein synthesis, but the mechanism of CrPyr influencing rumen fermentation remains unclear. This study aimed to combine 16S rDNA sequencing and metaproteomics technologies to investigate the microbial composition and function in rumen fluid samples taken from heat-stressed beef cattle treated with or without 60 g/d CrPyr.

Results: 16S rDNA sequencing revealed that there was no significant differences in the α-diversity indices between the two groups. By analyzing the expression profiles of 700 distinct proteins, we found that CrPyr administration increased the fatty acid β-oxidation, promoted the interconversion from pyruvate to phosphoenolpyruvate, acetyl-CoA and malate, up-regulated gluconeogenesis and citrate cycle metabolism, and promoted the biosynthesis of amino acids.

Conclusions: The increased generation of ATP during fatty acid β-oxidation or citrate cycle and the up-regulation synthesis of microbial protein in rumen of beef cattle treated with CrPyr, may help decreased oxidative stress, regulate energy metabolism, and further improve the rumen fermentation characteristic under heat stress.

Introduction

Ruminal microbiota is a large systematic microbial ecosystem that is composed of bacteria, archaea, protozoa, and fungi [1]. Microbial fermentation of feedstuff in the rumen produces volatile fatty acids (VFA) and microbial protein to provide the bulk of the energy and protein required by the host animal [2]. Hence, rumen microbes are essential for ruminant growth and health. However, many studies show that heat stress leads to severe defects in rumen function and variation in rumen microbial abundance and diversity [3-6]. Especially in southern China, due to the hot weather and high humidity, feedlot beef cattle finished in the summer months often suffer from heat stress. Therefore, the possibility of controlling the rumen microbial metabolism to achieve more efficient nutrient utilization under heat stress conditions has become an appealing concept for nutritionists.

Manipulation of feed additives such as antibiotics and vitamins is an efficient tool to relieve the negative impact of heat stress. However, a possible linkage between antibiotics in animal feed and the transmission of antimicrobial-resistant bacteria to humans has prompted researchers to explore other alternative approaches. As a new multifunctional nutrient, creatine pyruvate (CrPyr) contains pyruvic acid and creatine at a ratio of 40:60 [7], which both are natural body intermediate metabolites. This means that CrPyr can be used safely in the feed. Creatine and pyruvate are not only essential substrates for energetics but also critical antioxidants [8-10]. The results in our previous study showed that dietary supplementation with CrPyr improved stress resistance and increased antioxidant status and rumen fermentation of heat-stressed beef cattle [11]. It is implied that supplementing with CrPyr could be an
effective option to mitigate heat stress suffered by beef cattle. However, to date, detailed information about the changes in rumen microbial communities and related metabolic enzymes associated with the effects of CrPyr on rumen fermentation has not yet been published. Herein, an intensive study of rumen microbial diversity and microbial protein synthesis helps clarify the regulatory mechanism of CrPyr on rumen fermentation.

With the rapid development of bioinformatics, more researchers are starting to use meta-omics technology to define the rumen ecosystem’s ecology and its responses to changes in diet and rearing conditions [12]. Previous studies have shown that 16S rDNA sequencing was employed to characterize the composition and diversity of rumen microbes in Holstein heifers at elevated environmental temperatures and humidity, but little information about their function [3, 5]. While metaproteomics can provide the means to study the role of key enzymes involved in ruminant feed utilization efficiency under a particular set of conditions that dictate function. Analysis of the metaproteomes of the rumen microbial community would reveal details about microbial community activity, structure, function, and metabolic pathway transformations that are presently lacking [13]. Therefore, in this study, we combined 16S rDNA sequencing and metaproteomics technologies to understand the regulatory mechanism of CrPyr on rumen fermentation deeply.

Materials And Methods

2.1. Animal care

This experiment was approved by the Committee for the Care and Use of Experimental Animals at Jiangxi Agricultural University (JXAULL-20190017).

2.2. Animal treatments and experimental diets

Six ruminally cannulated Jinjiang steers (initial body weight = 405 ± 21 kg) were randomly allocated into two treatments. Jinjiang cattle are a breed of Chinese indigenous beef cattle bred in the northwest of the Jiangxi Province. The Jinjiang cattle received a CrPyr free basal diet (control group, CG) or a 60 g/d CrPyr supplemental basal diet (experiment group, EG) for 22 days. The purity of CrPyr is 99.9% (Hubei Ju Sheng Technology Co., Ltd., Wuhan, China). Our previous study results indicated that the Jinjiang cattle fed with 60 g/d CrPyr had a better resistance to heat stress [11]. Therefore, the present experiment selected a 60 g/d dose to explore the regulatory mechanism of CrPyr on rumen fermentation. The CrPyr was added to the concentrates, divided into two daily feeds (07:00 and 16:00). After the concentrate was fed, rice straw was given. All steers were housed in individual solid concrete floor pens in a closed cowshed, while clean, fresh water was available at all times. The composition and nutrient levels of the experimental diet were shown in Additional file 1: Table S1.

2.3. Measurement of temperature and humidity index, body temperature, and respiratory rate
The temperature and humidity index (THI) was measured according to Song et al. [14]. The thermohygrometer was suspended on the wall around the cattle's bodies on both sides of the experimental herd (approximately 1.5 m above the ground). The temperature and relative humidity at 08:00, 14:00, and 20:00 were recorded each day. Calculation of the average daily THI and the Livestock Weather Safety Index (LWSI) classifications for heat stress used the method of LCI [15]. Body temperature was measured via the rectum using a veterinary thermometer at 08:00, and their respiratory rate was measured via manual count at 08:30 and 14:00 until day 21 during the experimental period.

2.4. Samples collection

Samples of feed, feces, and rumen fluid were collected. All feces samples were collected from each steer on days 19 to 21. The feces were collected using a plastic bucket placed behind the cattle. The feces were thoroughly mixed, and 5% of the total feces were sampled once daily. After collection, the three days of fecal samples were mixed, and a subsample (300 g) was frozen at −20°C and used later to measure dry matter, ash, crude fat, neutral detergent fiber, and acid detergent fiber contents. The other subsample (100 g) was thoroughly mixed with 20 mL of 10% concentrated sulfuric acid and stored at −20°C until it could be analyzed for crude protein.

On day 21, rumen fluid samples were collected from the upper, middle, and lower sites in the rumen immediately post-feeding in the morning. Approximately 100 mL of rumen fluid was collected from each collection site. The rumen fluid was immediately measured for pH using a portable pH meter (HANNA Instruments, Cluj-Napoca, Romania). The rumen fluid samples were then filtered through four cheesecloth layers, and the samples were divided into three portions. The first 8 mL of rumen fluid was mixed with 2 mL of 25% (wt/vol) metaphosphoric acid and used for VFA analysis. One subsample (10 mL) of rumen fluid was mixed with 2 mL of H₂SO₄ (1% vol/vol) for determination of NH₃-N, and another subsample (10 mL) of rumen fluid was used for microbial crude protein (MCP), 16S rDNA sequencing and metaproteomics analysis. These samples were frozen at −20°C until analysis.

2.5. Chemical analyses

The VFA concentrations in the rumen fluid samples were determined using gas chromatography (Shimadzu GC-2014, Japan) equipped with a capillary column (Stabilwax, Restek, Bellefonte, PA, USA). The NH₃-N concentration was measured using a TU-1901 spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., China) according to a method described by Broderick and Kang [16]. The MCP production was determined according to the method of Makkar and Becker [17].

The feed and feces samples were dried at 60°C and ground through a 1-mm stainless steel screen. The dry matter, ash, and crude fat of the feed and fecal samples were determined according to methods 967.03, 924.05, and 920.39 of the AOAC [18], respectively. The neutral detergent fiber and acid detergent fiber were analyzed using an Ankom A200i Fiber Analyzer (ANKOM Technology Co., New York, NY, USA) according to the methods of Van Soest et al. [19]. The total nitrogen contents of the feed and feces samples were determined according to procedure 984.13 of the AOAC [18].
2.6. 16S rDNA sequencing analysis

2.6.1. DNA extraction and PCR amplification

According to manufacturer, microbial community genomic DNA was extracted from ruminal uid samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5’-ACTCCTACGGGAGGCAGCAG-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of 16S rRNA gene was performed as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and single extension at 72°C for 10 min, and end at 4°C. The PCR mixtures contain 5 × TransStart FastPfu buffer 4 μL, 2.5 mM dNTPs 2 μL, forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, TransStart FastPfu DNA Polymerase 0.4 μL, template DNA 10 ng, and finally ddH₂O up to 20 μL. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to manufacturer’s instructions and quantified using Quantus™ Fluorometer (Promega, USA).

2.6.2. Illumina MiSeq sequencing

Purified amplicons were pooled in equimolar and paired-end sequenced (2 ×300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP******).

2.6.3. Processing of sequencing data

The raw 16S rDNA gene sequencing reads were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap region is 0.2. Reads that could not be assembled were discarded; (iii) Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, exact barcode matching, 2 nucleotide mismatch in primer matching.

Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE (version 7.1, http://drive5.com/uparse/), and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the 16S rRNA database (eg. Silva 132/16s_bacteria) using confidence threshold of 0.7.
2.7. Meta-proteomics analysis

2.7.1 Protein extraction

The processed rumen fluid sample was centrifuged at 4°C for 5 minutes at 800 g to collect the supernatant. The supernatant was centrifuged at 7000 g for 20 min at 4°C, then BPP was added in the ratio of 1:10 to the resultant precipitate and ground with liquid nitrogen three times, and each grind lasted for 120 s. The solution was centrifuged at 12000 g for 20 min at 4°C, and supernatant was collected. The equal volume of Tris-saturated phenol was added and vortexed for 10 min at 4°C. The solution was centrifuged at 12000 g for 20 min at 4°C and the phenol phase was collected. The equal volume of BPP was added and vortexed for 10 min at 4°C. The solution was centrifuged at 12000 g for 20 min at 4°C and the phenol phase was collected. Five volumes of pre-cooled 0.1M ammonium acetate in methanol was added and precipitated protein at -20°C overnight. The supernatant was discarded by centrifugation, and the precipitate was washed twice with 90% acetone. Discard the supernatant by centrifugation and air dry the precipitate. The precipitate was re-suspended with lysis buffer (1% SDS, 8 M urea, cocktail), then sonicated for 2 min on ice. The lysate were centrifuged at 12000 g for 20 min at 4°C, and supernatants were collected to test the protein concentration in all samples. Protein concentrations were determined by the bicinchoninic acid (BCA) method.

2.7.2 Protein Digestion

Protein digestion was performed according to the standard procedure. Briefly, for each sample tube containing 100 μg protein, appropriate TEAB was added to the final concentration of 100 mM. Then, TCEP was added to the final concentration of 10 mM, and the tubes were incubated at 37°C for 60 min. Appropriate IAM was added to the final concentration of 40 mM and reacted for 40 min in dark. Add six volumes of cold acetone to the sample tube, and incubate the tube at -20°C for four h. The acetone was removed by centrifugation at 10000 g for 20 min, and precipitated protein was resuspended with 100 μL 100 mM TEAB Buffer. To each sample tube, according to the proportion 1:50 added the trypsin solution and incubated the tubes at 37°C overnight.

2.7.3 Peptide desalination and quantification

The peptides were vacuum dried, then resuspended with 0.1% TFA. Samples were desalted with HLB and vacuum dried. Peptide concentrations were determined by the Thermo Fisher Scientific peptide quantification kit (Thermo, Cat.23275). Loading buffer was added to each tube to prepare samples for mass spectrometry analysis, and the concentration of each sample was 0.25 μg/μL.

2.7.4 Mass spectrometry analysis

Experiments were performed on a Q Exactive mass spectrometer that was coupled with Easy-nLC 1200. Each peptide sample was injected for nanoLC-MS/MS analysis. The sample was loaded onto a C18-reversed phase column (75 μm × 25 cm, Thermo, USA) in buffer A (2% acetonitrile and 0.1% Formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% Formic acid) at a flow rate of
300 nL/min. The electrospray voltage of 1.8 kV versus the inlet of the mass spectrometer was used. The Exactive mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 350-1300) were acquired with a mass resolution of 70 K, followed by twenty sequential high energy collisional dissociation (HCD) MS/MS scans with a resolution of 17.5 K. In all cases, one microscan was recorded using the dynamic exclusion of 20 seconds.

2.7.5 Sequence Database Searching

MS/MS spectra were searched using ProteomeDiscoverer™ Software 2.2 software against the UniProt database (uniprot-taxonomy_3A171549_3A186802_3A191303_.fasta / LL_PEAKS_exported_proteins.fasta) as the following parameters. The highest score for a given peptide mass (best match to that predicted in the database) was used to identify parent proteins. The parameters for protein searching were set as follows: tryptic digestion with up to two missed cleavages, carbamidomethylation of cysteines as fixed modification, and oxidation of methionines and protein N-terminal acetylation as variable modifications. Peptide spectral matches were validated based on q-values at a 1% false discovery rate (FDR).

2.8. Statistical analysis

Statistical analysis of body temperature, respiratory rate, rumen fermentation, nutrient digestibility, and taxonomy were performed by independent sample t-test with SPSS (SPSS 2008) software. Mean values and standard error (SE) were reported, and differences were considered to be significant at $P < 0.05$.

Results

3.1. Temperature and humidity index

As shown in Fig. 1, there were three times daily changes in THI values in the morning (08:00), noon (14:00), and evening (20:00) during the experimental period. The average daily THI values during the experimental period were higher than 79 for all 22 experimental days.

3.2. Body temperature, respiratory rate, rumen fermentation, and nutrient digestibility

Dietary supplementation with CrPyr decreased the body temperature of beef cattle (Fig. 2a, $P < 0.05$), but did not affect the respiratory rate of beef cattle (Fig. 2b). Diet supplemented with CrPyr increased the ruminal pH value (Fig. 2c, $P < 0.05$) and MCP concentration (Fig. 2d, $P < 0.05$). No effect was observed on the contents of NH$_3$-N (Fig. 2e), total VFA (Fig. 2f), or individual VFA (Fig. 2g). As shown in Fig. 2h, CrPyr supplementation increased the crude fat digestibility ($P < 0.05$). No difference was found between groups for the digestibility of the dry matter, organic matter, crude protein, neutral detergent fiber, and acid detergent fiber.
3.3. Diversity of rumen fluid microbiota of beef cattle as revealed by 16S rDNA high-throughput sequencing

The high-throughput sequencing technology was used to investigate the effects of CrPyr on microbial communities in the rumen of beef cattle. The sequences were clustered into 1306 OTUs with a similarity of 97%. Among these, a total of 1098 OTUs were common, 109 OTUs were specific for the EG, and the CG had 99 unique OTUs (Additional file 2: Fig. S1). The rumen microbiota α-diversity of the two groups was evaluated by the Ace index, Chao1 index, Shannon index, and Simpson index (Additional file 3: Table S2). According to the result, there was no significant differences in the α-diversity index between the two groups.

3.4. Composition of rumen fluid microbiota of beef cattle as revealed by 16S rDNA sequencing and metaproteomics

16S rDNA sequencing and metaproteomics were combined to investigate the effects of CrPyr on both the structure and function of rumen fluid microbiota of beef cattle. 16S rDNA sequencing revealed that bacteria belonging to the phyla Bacteroidetes and Firmicutes comprised most (the average coverage was ~95.4%) of the total bacteria in the rumen fluid microbiota of the CG and EG. The remaining bacteria were mainly members of Actinobacteria, Spirochaetes, and Verrucomicrobiota (Fig. 3a). The abundance of Verrucomicrobiota was significantly different between the two groups \((P<0.05)\). At the genus level, the Rikenellaceae_RC9_gut_group was the dominant bacteria, and the other abundant genera were Prevotella, NK4A214_group, Christensenellaceae_R-7_group, Prevotellaceae_UCG-003, and Succiniclasticum (Fig. 3b).

Label-free quantification (LFQ) proteomics was applied to investigate the rumen fluid microbiota proteins of beef cattle. A total of 716,150 MS/MS spectra were generated from the six rumen fluid samples. Of these, 11,361 peptides (16.2%) could be identified and assigned to 3579 proteins (Additional file 4: Table S3). They included 2997 proteins that were quantified in the rumen fluid microbiota of EG and CG. The number of overlapping proteins between the two groups was 2153 (71.8%), while 373 and 206 community-specific proteins were unique to the rumen fluid microbiota in EG and CG, respectively, the number of quantified proteins without quantitative value (refers to a protein expressed in samples with less than 2/3 in both groups) was 265. Furthermore, in the 2153 overlapping proteins, 121 differentially expressed proteins (DEPs) were identified in the EG compared with the CG, of which 67 proteins were up-regulated and 54 proteins were down-regulated (Fig. 4b, \(P<0.05\)). Of the proteins identified, most were related to cell metabolism (3407). Others were related to organismal systems (188), human diseases (324), genetic information processing (612), environmental information processing (295), and cellular processes (125) (Fig. 4a).

The 16S rDNA sequencing and proteomic data revealed differences in the compositions of the dominant phyla. 16S rDNA sequencing revealed that the 16S rDNA relative abundances (abbreviated as 16SDA hereafter) of members of Bacteroidetes increased from 54.08 with CG to 56.89% with EG, the Firmicutes 16SDA decreased from 41.47 to 38.35% (Fig. 3a). Metaproteomics showed that the protein relative
abundances (abbreviated as PRA hereafter) of members of Bacteroidetes in total quantified proteins decreased from 74.15 with CG to 66.30% with EG, the Firmicutes PRA increased from 25.85 to 33.70% (Fig. 3c). At the genus level, the proteomic data showed that members of Prevotella were the dominant proteins, and the other abundant proteins were members of Bacteroides, Clostridium, Ruminococcus, and Alistipes (Fig. 3d).

3.5. Composition and functional classification of differentially expressed proteins as revealed by metaproteomics

The metaproteomics data showed that all 700 (373 unique in EG + 206 unique in CG + 121 differential overlapping, Additional file 5: Table S4). DEPs were members of Bacteroidetes and Firmicutes (Fig. 3e). The Bacteroidetes PRA was increased and the Firmicutes PRA was decreased significantly in the EG group than those in the CG group ($P < 0.05$). At the genus level, similar to the total quantified proteins (Fig. 3d), the members of Prevotella were also the dominant proteins in DEPs (Fig. 3f), and the other abundant proteins were Clostridium, Bacteroides, Flavonifractor, and Ruminococcus (Fig. 3f).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was used to explore the biological pathways for DEPs between the rumen fluid microbiota of EG and CG. Fig. 5a showed the KEGG pathway enrichment analysis of all DEPs. Fig. 5b showed the KEGG pathway enrichment analysis of all up-regulated DEPs, diet supplemented with CrPyr significantly enriched proteins involved in environmental information processing (HIF-1 signaling pathway, two-component systems); genetic information processing (ribosome, RNA degradation); human diseases (alzheimer disease, tuberculosis); metabolism (2-oxocarboxylic acid metabolism, biosynthesis of antibiotics, butanoate metabolism, carbon fixation in photosynthetic organisms, carbon metabolism, glycine, serine and threonine metabolism, glycolysis/gluconeogenesis, purine metabolism, pyruvate metabolism); organismal systems (longevity regulating pathway-worm). Fig. 5c showed the KEGG pathway enrichment analysis of all down-regulated DEPs, diet supplemented with CrPyr significantly decreased proteins involved in genetic information processing (ribosome, RNA polymerase); metabolism (alanine, aspartate and gyltamate metabolism, arginine biosynthesis, biosynthesis of antibiotics, butanoate metabolism, carbon fixation in photosynthetic organisms, carbon fixation pathway in prokaryotes, carbon metabolism, citrate cycle (TCA cycle), fructose and mannose metabolism, glycine, serine and threonine metabolism, glycolysis/gluconeogenesis, glyoxylate and dicarboxylate metabolism, nitrogen metabolism, pentose and glucuronate interconversions, pyruvate metabolism, valine, leucine and isoleucine degradation); organismal systems (GABAergic synapse, glutamatergic synapse).

Interactive Pathways Explorer (IPath) analysis was also used to visualize the mutual relationship of DEPs in metabolic (Fig. 6a) and microbial metabolism (Fig. 6b), on which red lines show up-regulated pathways, green lines show down-regulated pathways, blue lines show both up-regulated and down-regulated pathways. As shown in metabolic and microbial metabolism, the up-regulated pathways mainly including lipid metabolism, glycolysis/gluconeogenesis, and pyruvate metabolism.

3.6. Administration of CrPyr affected core enzymes related to key metabolism
3.6.1. Fatty acid metabolism

As shown in Fig. 7a and Additional file 6: Table S5, dietary supplemented with CrPyr up-regulated the Acyl-CoA dehydrogenase (EC: 1.3.8.1) from Lachnoclostridium sp., Lachnospiraceae bacterium, and Oscillibacter sp. but down-regulated this enzyme from Muribaculum. Exposure to CrPyr up-regulated the Acetyl-CoA C-acetyltransferase (ACAA) (EC: 2.3.1.9) from Oscillibacter sp.. Moreover, the 3-oxoacyl-[acyl-carrier-protein] synthase 2 (EC: 2.3.1.179) from Lentimicrobiaceae bacterium was up-regulated, and the 3-oxoacyl-[acyl-carrier-protein] reductase (EC: 1.1.1.100) from Prevotella sp. BP1-148 was down-regulated. The description of DEPs involved in fatty acid metabolism were showed in Additional file 6: Table S5.

3.6.2. Pyruvate metabolism

As shown in Fig. 7b, for the pyruvate metabolism pathway, 62 DEPs were identified in the EG compared with the CG, of which 47 proteins were up-regulated and 15 proteins were down-regulated. Of the 47 up-regulated proteins, most were pyruvate, phosphate dikinase (PPDK) (EC: 2.7.9.1) (17). Others were pyruvate:ferredoxin (Flavodoxin) oxidoreductase (6) and pyruvate-flavodoxin oxidoreductase (2) (EC: 1.2.7.1), phosphoenolpyruvate carboxykinase (ATP) (7) (EC: 4.1.1.49), and so on. The description of DEPs involved in pyruvate metabolism were showed in Additional file 7: Table S6.

3.6.3. Glycolysis / Gluconeogenesis

As shown in Fig. 7c, for the glycolysis / gluconeogenesis, 101 DEPs were identified in the EG compared with the CG, of which 75 proteins were up-regulated and 26 proteins were down-regulated. Except for EC: 1.2.7.1, EC: 4.1.1.49, EC: 1.2.7.11, which were also involved in pyruvate metabolism, the most up-regulated protein was glyceraldehyde-3-phosphate dehydrogenase (21, including 3 Type I glyceraldehyde-3-phosphate dehydrogenase (Fragment) ) (EC: 1.2.1.12). Others were fructose-1,6-bisphosphate aldolase, class II and its isozyme (7) and (EC: 4.1.2.13), phosphoglycerate kinase (5) (EC: 2.7.2.3), pyrophosphate–fructose 6-phosphate 1-phosphotransferase (4) (EC: 2.7.1.90), enolase (4) (EC: 4.2.1.11), and so on. The description of DEPs involved in glycolysis / gluconeogenesis were showed in Additional file 8: Table S7.

3.6.4. Citrate cycle (TCA cycle)

As shown in Fig. 7d, for the TCA cycle, 39 DEPs were identified in the EG compared with the CG, of which 29 proteins were up-regulated and 10 proteins were down-regulated. Except for EC: 1.2.7.1, EC: 1.2.7.11, EC: 4.1.1.49, EC: 6.4.1.1, which also involved in pyruvate metabolism, the most up-regulated protein was succinate dehydrogenase/fumarate reductase and its isozyme (4) (EC: 1.3.5.4; EC: 1.3.5.1). The description of DEPs involved in TCA cycle were showed in Additional file 9: Table S8.

3.6.5. Nitrogen metabolism and biosynthesis of amino acids

As shown in Fig. 7e, for the nitrogen metabolism pathway, 28 DEPs were identified in the EG compared with the CG, of which 11 proteins were up-regulated and 17 proteins were down-regulated. Of the 11 up-regulated proteins, including 8 glutamate dehydrogenase (GDH) (EC: 1.4.1.4) and 3 glutamine synthetase
(GS) (EC: 6.3.1.2). As shown in Fig. 7f, for the biosynthesis of amino acids, 94 DEPs were identified in the EG compared with the CG, of which 58 proteins were up-regulated and 36 proteins were down-regulated. The description of DEPs involved in nitrogen metabolism and biosynthesis of amino acids were showed in Additional file 10: Table S9, S10.

**Discussion**

The LWSI classifications for heat stress are as follows: normal, \( \leq 74 \); alert, \( 74 \leq \text{THI} \leq 79 \); danger, \( 79 \leq \text{THI} \leq 84 \); and emergency, \( \text{THI} \geq 84 \) [15]. The present study indicated that the experimental cattle were in a state of high heat stress due to the high temperature and humidity during the summer months. Consequently, to seek thermoregulation, animal employs a series of physiological and metabolic changes, including an increased rectal temperature, respiration rate, panting and open-mouth breathing, water consumption and a decrease in feed intake [20]. Body temperature is an excellent indicator of an animal's susceptibility to heat load, and the body core temperature of cattle in thermo-neutral conditions is maintained between 38 to 39.2°C [21]. In this study, in comparison to the control group, dietary supplementation with CrPyr significantly decreased the rectal temperature of cattle, which means CrPyr could relieve a heat stress-induced higher body temperature. Creatine is an osmotically active substance, and the osmotic effect of creatine might affect the thermoregulatory processes during exercise. Some research has suggested that, in humans, a potential mechanism involving increased extracellular fluid induced by added creatine may help the body deal with heat stress [22].

Rumen pH value and rumen bacterial populations are decreased during heat stress [3, 23]. Rumen pH value is the result of the combined effect of factors such as the VFA in rumen fluid, buffer salt in saliva, organic acid produced by metabolism, \( \text{NH}_3\)-N level, and emptying speed of rumen fluid. The production of \( \text{NH}_3\)-N in the rumen can increase rumen pH value. In the present study, CrPyr supplementation increased the rumen pH value. This might be attributed to the slow continuous release of ammonia from creatine. As previously reported, calcium pyruvate supplementation of goat diets could increase the content of VFA in the rumen [24, 25]. Moreover, creatine has been widely used as a nitrogen supplement in ruminant nutrition in the past few decades [26]. However, there was no significant difference in VFA or \( \text{NH}_3\)-N concentration for beef cattle fed CrPyr in heat stress. This may be due to that the VFA production induced by pyruvate and simultaneously slow continuous release of ammonia from creatine in the rumen were partly used by rumen microbes to synthesize proteins. Consistently, we found CrPyr increased the content of MCP in the present study.

The results in the present study showed that supplementing with CrPyr significantly increased the crude fat digestibility. Simultaneously, the IPath analysis showed that the administration of CrPyr enriched proteins involved in lipid metabolism (Fig. 6a), including those involved in fatty acid degradation and fatty acid biosynthesis, in the rumen fluid microbiota of beef cattle. The present study was the first metabolic evidence to demonstrate that CrPyr promotes the lipid metabolism of the rumen fluid microbiota of beef cattle. The metaproteomics screening in the present study detected the expression of short-chain acyl-CoA dehydrogenase (SCAD) and ACAA (Additional file 11: Fig. S2; Additional file 6: Table
S5) were significantly up-regulated in the CrPyr supplemented group compared with the control group. Acyl-CoA dehydrogenase catalyzes the initial rate-limiting step, ACAA catalyzes the last step of the mitochondrial fatty acid β-oxidation. They are all key enzymes of the fatty acid oxidation [27, 28]. In the present study, the up-regulation of SCAD from *Lachnoclostridium sp.*, *Lachnospiraceae bacterium*, *Oscillibacter sp.* catalyze the dehydrogenation of Hexanoyl-CoA and Butanoyl-CoA. The up-regulation of ACAA from *Oscillibacter sp.* catalyzes the reaction of 3-oxo-hexanoyl-CoA and CoA to form Acetyl-CoA and Butanoyl-CoA, and the formation of two molecules of acetyl-CoA from acetoacetyl-CoA. Then, acetyl-CoA enter into the TCA cycle provides energy for metabolism. The above SCAD and ACAA catalyzed reactions could promote fatty acid degradation, which may explain the increased digestibility of crude fat in the CrPyr supplemented group. On the other hand, the ATP generation during fatty acid β-oxidation may help decrease oxidative stress, regulate energy metabolism, and further improve the rumen fermentation characteristic under heat stress.

Pyruvate is the intermediate-product of carbohydrate fermentation by rumen microorganisms [9]. Our metaproteomics analyses showed that the administration of CrPyr significantly up-regulated the pyruvate metabolism and glycolysis gluconeogenesis pathway. Phosphoenolpyruvate (PEP)/pyruvate interconversion is a major metabolic point in glycolysis and gluconeogenesis [29]. In the case of the pyruvate-to-PEP conversion, the reaction proceeds through diversified metabolic reactions. For instance, in propionic acid bacteria, PPDK is the primary enzyme that directly converts pyruvate to PEP in one step [30]. However, in most mammals, plants, and microorganisms, the reaction proceeds by two steps, catalyzed by pyruvate carboxylase and PEP carboxykinase [31]. In this study, the pyruvate to PEP conversion was promoted, proving that the PPDK was significantly enriched (including 17 up-regulated PPDK and 7 down-regulated PPDK from different bacteria). Besides, the pyruvate carboxylase subunit B from *Prevotella ruminicola*, and the PEP carboxykinase (ATP) from *Parabacteroides distasonis*, *Prevotellaceae bacterium MN60*, *Pseudobutyrivibrio ruminis*, *Prevotella sp. tf2-5*, *Prevotella bryantii*, *Lachnospiraceae bacterium KH1T2* were up-regulated. Moreover, in pyruvate metabolism, we also found that the conversion of pyruvate to acetyl-CoA or (S)-malate was promoted in the CrPyr administration group. In several microorganisms, pyruvate-flavodoxin oxidoreductase is responsible for acetyl-CoA formation from pyruvate in a single step [32]. Our metaproteomics analysis showed that the up-regulated enzymes involved in the pyruvate to acetyl-CoA conversion, including 13 pyruvate-flavodoxin oxidoreductase and its isozymes from different bacteria, 2-oxoglutarate ferredoxin oxidoreductase subunit alpha from *Prevotella sp. khp7*, and 2-oxoglutarate ferredoxin oxidoreductase subunit beta from *Prevotella sp. tf2-5*. Generally, microbes have five metabolic pathways involved in malate biosynthesis. Two of these pathways take pyruvate as the metabolic starting point: I) pyruvate is first converts to oxaloacetate via the pyruvate carboxylase, and then malate dehydrogenase converts oxaloacetate to malate; II) pyruvate is directly converted to malate via malic enzyme in one step [33]. In the present study, CrPyr administration up-regulated the malate dehydrogenase from *Bacteroides xylanolyticus*, NAD-dependent malic enzyme from *Clostridium homopropionicum DSM 5847*, and allosteric NADP-dependent malic enzyme from *Prevotella ruminicola*. 
The conversion of pyruvate to PEP is the first and rate-limiting step of gluconeogenesis. The up-regulation of PPDK, pyruvate carboxylase subunit B, and PEP carboxykinase (ATP) promoted the pyruvate-to-PEP conversion in CrPyr supplemented group. Meanwhile, several other enzymes involved in gluconeogenesis were up-regulated, including 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphate aldolase, pyrophosphate–fructose 6-phosphate 1-phosphotransferase, glucose-6-phosphate isomerase, phosphoglucomutase, aldose 1-epimerase. Of those enzymes, the glyceraldehyde-3-phosphate dehydrogenase was most enriched, with 21 up-regulated and 6 down-regulated proteins from different bacteria. These up-regulated enzymes implied that dietary supplemented with CrPyr promoted the gluconeogenesis of rumen microorganism.

The acetyl-CoA generated from fatty acid β-oxidation and pyruvate metabolism could enter the TCA cycle, promote the TCA cycle metabolism and provide energy. Consistent with this, we found that 29 proteins were up-regulated and 10 proteins were down-regulated in TCA cycle pathway. The up-regulation of TCA cycle metabolism might help to produce more α-ketoglutarate. When ammonia and α-ketoglutarate are in a certain ratio, the efficiency of MCP synthesis can be optimized without causing the accumulation of ammonia or α-ketoglutarate. This is rumen energy-nitrogen equilibrium [34]. In rumen, ammonium is the preferred nitrogen source for microbial growth. Creatine, as an important nitrogen-containing compound in protein and energy metabolism, has been widely used as a nitrogen supplement in ruminant nutrition due to its slow continuous release of ammonia [8, 26]. The results from nitrogen metabolism indicated that CrPyr administration led to 8 up-regulated bacterial reduced NADPH specific GDH (EC: 1.4.1.4), and 13 down-regulated bacterial NADPH specific GDH. We also found that 3 up-regulated and 3 down-regulated bacterial GS (EC: 6.3.1.2), and one down-regulated bacterial glutamate synthase (GOGAT) (EC: 1.4.1.13). The GDH and GS-GOGAT pathways are two classic routes for ammonia assimilation in bacteria [35]. In the GDH pathway, the NADH specific GDH serves to degrade glutamate:

\[ \alpha\text{-ketoglutarate} + \text{NADPH} + \text{NH}_3 \xrightarrow{\text{NADPH-GDH}} \text{NADP} + \text{glutamate} \]

[36]. In the GS-GOGAT pathway, GS is a ubiquitous enzyme that catalyzes the ATP-dependent amidation glutamate to generate glutamine, GOGAT catalyzes the reductive transfer of the amide group of glutamate. In this study, the GDH pathway appears to be the predominant route of ammonia assimilation. We further analyzed the DEPs involved in the biosynthesis of amino acids, of which 58 proteins were up-regulated and 36 proteins were down-regulated, these results showed that CrPyr was helpful to amino acid biosynthesis of bacteria in rumen fluid.

Conclusions
In conclusion, CrPyr administration increased the digestibility of crude fat through promoting the fatty acid β-oxidation of rumen bacteria, and up-regulated gluconeogenesis through increasing the production of PEP. The acetyl-CoA generated from fatty acid β-oxidation and pyruvate metabolism promote the TCA cycle metabolism, and might help to produce more α-ketoglutarate. The α-ketoglutarate, together with the NH$_3$ released by creatine, promoted the synthesis of MCP in the rumen. The increased production of ATP and the up-regulation synthesis of MCP in rumen, may help decrease oxidative stress, regulate energy metabolism, and further improve the rumen fermentation characteristic of beef cattle under heat stress (Fig. 8).

**Abbreviations**

CrPyr: Creatine pyruvate; PEP: Phosphoenolpyruvate; VFA: Volatile fatty acids; CG: Control group; EG: Experimental group; THI: Temperature and humidity index; LWSI: Livestock Weather Safety Index; MCP: Microbial crude protein; OTUs: Operational taxonomic units; LFQ: Label-free quantification; 16SDA: 16S rDNA relative abundances; PRA: Protein relative abundances; DEPs: differentially expressed proteins; KEGG: Kyoto Encyclopedia of Genes and Genomes; IPath: Interactive Pathways Explorer; ACAA: Acetyl-CoA C-acetyltransferase; SCAD: Short-chain acyl-CoA dehydrogenase; PPDK: Pyruvate, phosphate dikinase; GDH: Glutathione dehydrogenase; GS: Glutamine synthetase; GOGAT: Glutamate synthase

**Declarations**

**Acknowledgments**

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**Authors’ contributions**

MQ, YZ, and XZ designed the experiments. YL, LL, KM and GL performed the experiments. YL and LL analysed the data. YL wrote the main manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

Raw sequence reads for all samples described above were deposited into the NCBI Sequence Read Archive (SRA) database (accession number, SRP******) and ProteomeXchange database (project number, PRJNA******).
Ethics approval

All of the experiments were discussed and approved by the Committee for the Care and Use of Experimental Animals at Jiangxi Agricultural University (JXAULL-20190017).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Figures**
Figure 1

Temperature-humidity index in the house of the beef cattle. THI, temperature and humidity index
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Figure 2

Effect of creatine pyruvate on body temperature (a), respiratory frequency (b), rumen fermentation parameter (c–g), and nutrient digestibility (h) of beef cattle. The rumen fermentation parameter including ruminal pH (c), the concentration of microbial crude protein (MCP) (d), NH3-N (e), and total VFA (f), the proportion of individual VFA (g). CG = control group; EG = experimental group, diet supplemented with 60 g/d CrPyr. n = 3 per group. *P < 0.05, **P < 0.01.
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Figure 3

Phylogenetic classification and metaproteomics analyses of rumen fluid microbiota of beef cattle fed a CrPyr supplementation diet. a and b show phylum-level a and genus-level b rumen fluid community composition based on Illumina sequences of 16S rDNA amplicons (V3-V4 region). c and d show relative abundances of total identified proteins in rumen fluid assigned to bacterial phyla c and genera d. e and f show relative abundances of differentially expressed proteins in rumen fluid assigned to bacterial phyla e.
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Figure 4

Metaproteomics analyses of proteins in the rumen fluid microbiota of beef cattle fed a CrPyr supplementation diet. a KEGG classification. b Volcano plots of differentially expressed proteins between EG and CG. EG = experimental group, diet supplemented with 60 g/d CrPyr; CG = control group. Each dot represents the mean expression level (n = 3) of a single protein. The yellow dots indicate the significantly up-regulated proteins at P < 0.05 level, the red dots indicate the significantly up-regulated proteins at P < 0.01 level, the light blue dot indicates the significantly down-regulated proteins P < 0.05 level, the blue dot indicates the significantly down-regulated proteins P < 0.01 level.
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Figure 5

KEGG enrichment of differentially expressed proteins. a KEGG enrichment analysis of all differentially expressed proteins. b KEGG enrichment analysis of all up-regulated differentially expressed proteins (experimental group vs control group, EG vs CG). c KEGG enrichment analysis of all down-regulated differentially expressed proteins (experimental group vs control group, EG vs CG). CP = Cellular
Figure 5

KEGG enrichment of differentially expressed proteins. a KEGG enrichment analysis of all differentially expressed proteins. b KEGG enrichment analysis of all up-regulated differentially expressed proteins (experimental group vs control group, EG vs CG). c KEGG enrichment analysis of all down-regulated
differentially expressed proteins (experimental group vs control group, EG vs CG). CP = Cellular Processes; EIP = Environmental Information Processing; GIP = Genetic Information Processing; HD = Human Diseases; M = Metabolism; OS = Organismal Systems.

Figure 6

Interactive Pathways Explorer (iPath) analysis. a Metabolic. b Microbial metabolism. Red lines show up-regulated pathways, green lines show down-regulated pathways, blue lines show both up-regulated and
Figure 6

Interactive Pathways Explorer (iPath) analysis. a Metabolic. b Microbial metabolism. Red lines show up-regulated pathways, green lines show down-regulated pathways, blue lines show both up-regulated and down-regulated pathways.
Figure 7

Protein identity and regulation involved in several important pathway in rumen fluid samples of beef cattle fed with a CrPyr supplementation diet (experimental group vs control group, EG vs CG). a fatty acid metabolism. b pyruvate metabolism. c glycolysis / gluconeogenesis. d citrate cycle (TCA cycle). e nitrogen metabolism. f biosynthesis of amino acids.
Figure 7

Protein identity and regulation involved in several important pathway in rumen fluid samples of beef cattle fed with a CrPyr supplementation diet (experimental group vs control group, EG vs CG). a fatty acid metabolism. b pyruvate metabolism. c glycolysis / gluconeogenesis. d citrate cycle (TCA cycle). e nitrogen metabolism. f biosynthesis of amino acids.
Figure 8

Proposed model of creatine pyruvate relieving heat stress in rumen of beef cattle
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