Metabolome profiling of plasma reveals different metabolic responses to acute cold challenge between Inner-Mongolia Sanhe and Holstein cattle

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ABSTRACT

Low-temperature conditions influence cattle productivity and survivability. Understanding the metabolic regulations of specific cattle breeds and identifying potential biomarkers related to cold challenges are important for cattle management and optimization of genetic improvement programs. In this study, 28 Inner-Mongolia Sanhe and 22 Holstein heifers were exposed to −25°C for 1 h to evaluate the differences in metabolic mechanisms of thermoregulation. In response to this acute cold challenge, altered rectal temperature was only observed in Holstein cattle. Further metabolome analyses showed a greater baseline of glycolytic activity and mobilization of AA in Sanhe cattle during normal conditions. Both breeds responded to the acute cold challenge by altering their metabolism of volatile fatty acids and AA for gluconeogenesis, which resulted in increased glucose levels. Furthermore, Sanhe cattle mobilized the citric acid cycle activity, and creatine and creatine phosphate metabolism to supply energy, whereas Holstein cattle used greater AA metabolism for this purpose. Altogether, we found that propionate and methanol are potential biomarkers of acute cold challenge response in cattle. Our findings provide novel insights into the biological mechanisms of acute cold response and climatic resilience, and will be used as the basis when developing breeding tools for genetically selecting for improved cold adaptation in cattle.

Key words: acute cold challenge, metabolic profile, Inner-Mongolia Sanhe, Holstein

INTRODUCTION

Environmental temperature is a physical factor that can influence animal welfare and productivity. Animals have an optimum function of biological processes under the thermoneutral zone (Brouček et al., 1991). Exposure to temperatures out of the thermoneutral zone results in behavioral and physiological stress responses (e.g., changes in respiration rate and body temperature; Sefjian et al., 2018; Amamou et al., 2019) and biochemical changes (e.g., different hormones and metabolic profiles; Verbeek et al., 2012; Lian et al., 2017) to maintain the homeostasis. Animals can experience cold stress when the environmental temperature reduces below the lower critical temperature.

Cold stress is a major consequence of climate change, especially in the Northern hemisphere (Kang et al., 2016; Hristov et al., 2018), and results in economic losses and welfare issues in the worldwide livestock industry (Nguyen et al., 2016; Toghiani et al., 2020). Therefore, lower ambient temperatures during the winter season are a concern for cattle production systems in areas with harsh winters because it results in decreased growth (Kang et al., 2016), production (Young, 1981), and reproductive performance (Sasaki et al., 2016), as well as a compromised immune response (Kang et al., 2016). Furthermore, other climatic variables such as wind, snow, and rain also aggravate the effects of cold stress in cattle. For instance, with rainfall, the effects of cold stress are more severe due to the greater heat loss as a result of evaporation (Schütz et al., 2010). Therefore, investigating the biological mechanisms of response to cold conditions are essential for developing strategies to minimize the unfavorable effects of climatic adaptation.

Metabolomics is an emerging field in the area of systems biology, with metabolic profiling capturing the final biological products of the animals’ bodies during
We used the 1H nuclear magnetic resonance (1H NMR) platform in combination with multivariate statistical analyses to determine the differences in metabolic profiles and metabolic adaptation under acute cold challenge between Sanhe and Holstein cattle. These results contribute to a better understanding of the biological mechanisms of response to cold exposure, which is essential for the implementation of genetic selection strategies for improving climatic adaptation in cattle.

**MATERIALS AND METHODS**

**Animals and Sample Collection**

All the use of animals for the current research was approved by the Committee on Ethics of Animal Experimentation from the China Agricultural University (Beijing, China). The experiment was performed at the Xiertala Cattle Breeding Farm (Hailaer, Inner Mongolia, China). Fifty (28 Sanhe and 22 Holstein) non-pregnant heifers with similar age (12.7 ± 2.3 mo and 12.5 ± 2.1 mo, for Sanhe and Holstein, respectively) and weight (392.3 ± 52.1 kg and 376.3 ± 49.3 kg, for Sanhe and Holstein, respectively) were included in the experiment. All animals were housed in cowshed during the winter season (before and after the acute cold challenge) and fed the same diet of TMR at 0500, 1100, and 1700 h. Ad libitum water was available during the whole experiment.

This current study is an extension of a previous study (Hu et al., 2021), which only evaluated a smaller sample size of a single breed (Inner-Mongolia Sanhe cattle) and employed different analytical methods. The influence of acute cold challenge on the metabolome between the 2 breeds was investigated by transferring all animals outdoors, and exposing them to approximately −25°C (±0.9°C, measured using a digital Temperature Humidity Meter with a precision of 0.1°C; testo635–2, Shanghai Minyi Electronics Co. Ltd.) for 1 h, after housing them at 5°C (±1.2°C) for 15 h, during the winter season (Figure 1). Rectal temperature (RT) was measured immediately before and after cold challenge using a digital thermometer with a precision of 0.1°C (MC-347, Omron Dalian Co. Ltd.). Blood samples (10 mL) with anticoagulant of heparin sodium were collected before and after cold exposure. Blood samples were centrifuged at 1,400 × g for 10 min at room temperature to obtain the upper plasma. The plasma samples were stored at −80°C until performing the metabolomic analyses.

**1H Nuclear Magnetic Resonance Measurement**

Metabolic profiles were derived from 1H NMR spectroscopy. All plasma samples were pretreated as previously described by Beckonert et al. (2007). In brief, samples were centrifuged at 14,500 × g for 15 min, and the upper layer was transferred to a 0.5-mL 3 KDa ultrafiltration filter (Merck & Co. Inc.). Subsequently, the samples were centrifuged at 14,500 × g for 45 min and a 450-μL aqueous layer was collected into a clean 2-mL centrifuge tube, followed by adding 50 μL of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) standard solution (Anachro Technologies Inc.). The mixture was transferred to a 5-mm NMR tube. Spectra were collected using an Agilent DD2 600 MHz spectrometer equipped with a triple-resonance cryoprobe. The first increment of a two-dimensional 1H nuclear Overhauser effect spectroscopy pulse sequence was used for the acquisition of 1H NMR data and suppressing the solvent signal. Experiments used a 100-ms mixing time, along with a 990-ns presaturation (~80 Hz gammaB1), and spectra were collected at 25°C, with 128 scans over a period within 15 min.
Data Preprocessing and Relative Quantification

The processing module in the Chenomx NMR Suite 8.1 software (Chenomx Inc.) was used to carry out the automatically zero-filled and Fourier transform of those collected free induction decay signals. The data were phased and baseline corrected. Differently from our previous study (Hu et al., 2021), the relative quantification method was used to capture the metabolic profiles due to the lower cost and time efficiency for the analyses. All samples were analyzed in the range of 0.00 to 10.00 ppm with 0.04 ppm as segmental integration unit after exclusion of data among 4.660 to 5.000 ppm (water peak), 0.568 to 0.680 ppm (DSS peak), 1.723 to 1.790 ppm (DSS peak), and 2.871 to 2.940 ppm (DSS peak), where DSS is the internal standard. Subsequently, integration bins (named chemical shifts in the following text) were normalized by weight across all parallel samples and exported to Excel format files.

Metabolome Analyses

We first analyzed the metabolome data sets of 2 breeds before exposure to acute cold challenge. Then, we compared the difference of metabolomes within each breed before and after acute cold exposure based on the analysis protocol for paired metabolome data, as described in our previous study (Hu et al., 2021). Log-transformation and Pareto scaling were used to approximate a Gaussian distribution and make the chemical shifts being equally weighted. Principal component analysis (PCA) was carried out for investigating separation or clustering trends, whereas partial least squares discriminant analysis (PLS-DA) was performed for selecting differential chemical shifts. The model quality of PLS-DA was determined by 10-fold cross-validation (9 groups for the training sets and one for the test set in each validation) with $R^2$ and $Q^2$ parameters, whereby $R^2$ indicates the goodness of fit score, and $Q^2$ quantifies the score of reliable prediction, as well
as 1,000 permutation tests to avoid overfitting (Xiao et al., 2019). Bins with variable importance in projection (VIP) scores >1 were identified as differential chemical shifts (Pérez-Enciso and Tenenhaus, 2003; Dervishi et al., 2017). In addition, Student’s t-test (Student’s paired t-test for paired data) was also used to select differential chemical shifts, with the standard of P-value <0.05 considered as statistically significant. All data sets were processed using the MetaboAnalyst online software (version 4.0, www.metaboanalyst.ca/faces/home.xhtml).

**Metabolic Pathway Analyses**

To identify differential metabolites responding to low temperature, all chemical shifts with VIP score >1 in PLS-DA or P-value <0.05 based on Student’s t-test (Student’s paired t-test for paired data) were annotated by using the Chenomx metabolite database (Chenomx Inc., https://www.chenomx.com/). Enrichment and pathway analyses were conducted with identified differential metabolites using the MetaboAnalyst software (version 4.0, www.metaboanalyst.ca/faces/home.xhtml). For this process, we selected the Kyoto Encyclopedia of Genes and Genomes (KEGG) database of Bos taurus (cattle) as the pathway library (https://www.genome.jp/kegg/). The algorithm for pathway enrichment analysis was a hypergeometric test, and the relative betweenness centrality was used to conduct the pathway topological analyses. The functional enrichment and pathway results with \( P < 0.05 \) were considered significant. Besides, the pathway impact value, which was calculated as the sum of the importance measures of the matched metabolites normalized by all metabolites in each pathway (Xia and Wishart, 2010), was used to identify the pivotal pathways, and we set 0.15 as the threshold.

**Identification of Potential Biomarkers for Acute Cold Challenge in Cattle**

Metabolites that met the thresholds of VIP value >1 in the PLS-DA analysis and \( P\)-value <0.05 in Student’s paired t-test in both breeds were selected for further analyses. Plasma concentrations of these metabolites were quantified using the absolute quantification method described in our previous study (Hu et al., 2021). Cluster analyses were performed using the HemI 1.0 package (version 1.0, Heatmap Illustrator) to verify whether they can accurately distinguish individuals before and after acute cold challenge. Moreover, the receiver operating characteristic curve analysis was performed using the SAS software (version 9.2, SAS Institute Inc.), and the area under the curve (AUC) was derived to determine the discriminatory power (Tian et al., 2015). The guide for assessing the best biomarker based on its AUC was as follows: excellent, AUC values between 0.9 and 1.0; good, AUC values between 0.8 and 0.9; fair, AUC values between 0.7 and 0.8; poor, AUC values between 0.6 and 0.7; and fail, with AUC values between 0.5 and 0.6 (Dervishi et al., 2017). Student’s paired t-tests were carried out to verify which biomarkers significantly differed before and after the acute cold challenge.

**ELISA for Metabolic Enzymes**

Based on the results of enrichment and pathway analyses, as well as the networks among differential metabolites, 10 metabolic enzymes (acetyl-CoA, A-CoA; acyl-CoA synthetase, ACS; alcohol dehydrogenase; branched-chain AA aminotransferase, BCAT; citrate synthase, CS; glucose-6-phosphatase, G-6-Pase; 3-hydroxyisobutryrate dehydrogenase, HIBADH; isocitrate dehydrogenase; pyruvate kinase; glutamine synthetase, GS) were selected for evaluating their activity in plasma. Nine molecules (A-CoA, ACS, alcohol dehydrogenase, BCAT, CS, G-6-Pase, HIBADH, isocitrate dehydrogenase, and pyruvate kinase) were detected using the commercial ELISA kits (Beijing Jinhaikeyu Biotechnology Co. Ltd.), whereas GS was determined by the method of spectrophotometry (Beijing Jinhaikeyu Biotechnology Co. Ltd.).

**Statistical Analyses**

Student’s t-test was used to evaluate the difference in RT, metabolites, and metabolic enzymes between Sanhe and Holstein animals. Student’s paired t-test was used to compare the difference of the above variables before and after acute cold challenge within each breed. The results are presented as mean ± standard deviation, and \( P\)-value <0.05 was considered as the significance level.

**RESULTS**

**Rectal Temperature Responses to Acute Cold Challenge in Sanhe and Holstein Cattle**

As shown in Figure 2A, we found no significant difference in RT between Holstein (39.1 ± 0.3°C) and Sanhe (39.3 ± 0.2°C) cattle when the environmental temperature was 5°C which can be regarded as thermoneutral zone condition (\( P > 0.05 \)). The RT significantly increased in Holstein cattle after exposure to −25°C
for 1 h (39.1 ± 0.3°C versus 39.4 ± 0.2°C, \( P < 0.001 \)). However, RT did not increase in Sanhe cattle after exposure to cold temperatures (39.3 ± 0.2°C versus 39.3 ± 0.3°C, \( P > 0.05 \)). These results indicate that Sanhe and Holstein cattle breeds might have different abilities to respond to short-term exposure to cold temperatures.

**Metabolome Overview of the Two Breeds**

Typical \(^1\)H NMR spectra of plasma samples from Sanhe and Holstein cattle (Sanhe before acute cold challenge, Sanhe after acute cold challenge, Holstein before acute cold challenge, and Holstein after acute cold challenge).
cold challenge) are shown in Figures 2B and 2C, respectively. There were 238 chemical shifts obtained by relative quantification, which were used for further analyses. The PLS-DA score plot showed a clustering trend between the 2 breeds not only before, but also after the cold challenge (Figure 2D), suggesting that plasma metabolic profiles are different between Sanhe and Holstein.

**Differences of Plasma Metabolomes and Metabolic Enzymes Between Sanhe and Holstein Cattle**

The metabolome data sets of the 2 breeds before acute cold challenge were used to perform the PCA (Supplemental Figure S1A; https://doi.org/10.6084/m9.figshare.20010488.v1; Hu, 2022a) and PLS-DA (Figure 3A) analyses. A separation in the plasma metabolomes between Sanhe and Holstein cattle can be observed in the score plot of PLS-DA (Figure 3A). The current PLS-DA model presented high values of R²Y (0.93) and Q²Y (0.75) in the cross-validation, as well as a P < 0.001 in the permutation tests (Supplemental Figure S1B and S1C). A total of 26 differential chemical shifts were selected according to their VIP values in the PLS-DA model (VIP > 1). The top 15 are shown in Figure 3B). In addition, 42 chemical shifts significantly differed between the 2 breeds (P < 0.05). As shown in Table 1, 20 differential metabolites were identified, and they are significantly involved in the pathways “glycine, serine, and threonine metabolism,” “D-glutamine and D-glutamate metabolism,” “alanine, aspartate, and glutamate metabolism,” “valine, leucine, and isoleucine biosynthesis,” and “glyoxylate and dicarboxylate metabolism” (Figure 3C; Supplemental Table S1; https://doi.org/10.6084/m9.figshare.20010512.v1; Hu, 2022d). The results of Student’s t-test for 10 metabolic enzymes are shown in Figure 3D and Supplemental Table S4 (https://doi.org/10.6084/m9.figshare.20010533.v1; Hu, 2022g), in which the activities of CS, G-6-Pase, and HIBADH were significantly different in the plasma of the 2 breeds.

**Differences of Plasma Metabolomes and Metabolic Enzymes Before and After Acute Cold Challenge in Sanhe Cattle**

Seventy-nine out of the 238 chemical shifts were pre-selected if 1 increased or decreased in ≥70% of animals after cold exposure. Further analyses using the subset of metabolomes showed clustering trends in both PCA (Supplemental Figure S2A; https://doi.org/10.6084/m9.figshare.20010500.v1; Hu, 2022b) and PLS-DA (Figure 4A). The R²Y and Q²Y in cross-validation were 0.85 and 0.62 (Supplemental Figure S2B), respectively, and the permutation tests displayed P < 0.001 (Supplemental Figure S2C). As shown in Figure 4B, 11 differential chemical shifts were observed with the threshold of VIP value >1 in the PLS-DA. Moreover, 27 chemical shifts were significantly different before and after acute cold challenge in Sanhe cattle (P < 0.05). Overall, 16 differential metabolites were identified by comparing the original spectra of ¹H NMR and the Chenomx database (Table 1). The pathway analysis highlighted that “D-glutamine and D-glutamate metabolism,” “valine, leucine, and isoleucine biosynthesis,” and “alanine, aspartate, and glutamate metabolism” were significantly involved in cold-induced responses in Sanhe cattle (Figure 4C; Supplemental Table S2; https://doi.org/10.6084/m9.figshare.20010515.v1; Hu, 2022e). When exposed to cold temperatures, only the activity of GS significantly increased (P < 0.05; Figure 4D; Supplemental Table S4). The levels of BCAT and A-CoA were also altered by cold exposure, but they were not statistically significant (P < 0.10).

**Differences of Plasma Metabolomes and Metabolism Enzymes Before and After Acute Cold Challenge in Holstein Cattle**

In Holstein cattle, 104 out of the 238 chemical shifts were found to be increased or decreased in more than 70% of the individuals. Score plots of PCA (Supplemental Figure S3A; https://doi.org/10.6084/m9.figshare.20010509.v1; Hu, 2022c) and PLS-DA (Figure 5A) demonstrated that the Holstein animals underwent metabolic changes during the acute cold challenge. Good performance of current PLS-DA model was observed (R²Y = 0.86, Q²Y = 0.56, and P-value <0.001; Supplemental Figure S3B and S3C). Twenty-two differential chemical shifts were selected with VIP value >1 (Figure 5B). In addition, 39 out of the 104 chemical shifts were significantly altered after being exposed to −25°C for 1 h (P < 0.05), and also identified as differential chemical shifts. Lastly, 17 differential metabolites were associated with acute cold challenge in Holstein cattle (Table 1), and mainly enriched for the “D-glutamine and D-glutamate metabolism,” “valine, leucine, and isoleucine biosynthesis,” “arginine and proline metabolism,” “alanine, aspartate, and glutamate metabolism,” and “phenylalanine, tyrosine, and tryptophan biosynthesis” pathways (Figure 5C; Supplemental Table S3; https://doi.org/10.6084/m9.figshare.20010530.v2; Hu, 2022f). Furthermore, we found that the activities of CS and G-6-Pase significantly increased after acute cold exposure in Holstein cattle (P < 0.05) (Figure 5D; Supplemental Table S4), and the activity of ACS was slightly higher after exposure to the acute cold challenge (P < 0.10).
Differences of Metabolic Responses on Acute Cold Challenge Between Sanhe and Holstein Cattle

To comprehensively understand the differences in the metabolic responses of acute cold exposure between Sanhe and Holstein, Venn diagrams were used to identify common and specific features for these breeds. In total, we found 16 and 17 differential metabolites in Sanhe after versus Sanhe before acute cold challenge, and Holstein after versus Holstein before acute cold challenge, respectively, in which 11 shared features were observed in 2 comparisons (Figure 6A).

Figure 3. The differential metabolites and their pathways between Sanhe and Holstein. (A) Scores plot of partial least squares discriminant analysis of metabolic profiling between Sanhe (SB) and Holstein (HB) before acute cold challenge. (B) Top 15 chemical shifts with higher variable importance in projection (VIP) scores. (C) The results of pathway analysis. (D) Key enzymes related to metabolic pathways. CS = citrate synthase; G-6-Pase = glucose-6-phosphatase; HIBADH = 3-hydroxyisobutyrate dehydrogenase. Student’s t-test was used to analyze the difference between Sanhe and Holstein. Student’s t-test was used in the results presented in (D). Values are presented as mean ± SD.
Table 1. Differential metabolites were identified in 3 comparisons of Sanhe and Holstein [Sanhe before (SB) versus Holstein before (HB) cold exposure; SB versus Sanhe after (SA) cold exposure; HB versus Holstein after (HA) cold exposure]

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pathway</th>
<th>SB vs. HB</th>
<th>Shift</th>
<th>VIP</th>
<th>P-value</th>
<th>2-log (FC)</th>
<th>SA vs. SB</th>
<th>Shift</th>
<th>VIP</th>
<th>P-value</th>
<th>2-log (FC)</th>
<th>HA vs. HB</th>
<th>Shift</th>
<th>VIP</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>2-Hydroxybutyrate</td>
<td>Lipid</td>
<td>1.18(s)</td>
<td>1.39</td>
<td>0.44</td>
<td>0.05</td>
<td>1.94</td>
<td>2.42(d), 2.30(s), 1.18(s)</td>
<td>1.94</td>
<td>&lt;0.001</td>
<td>-0.14</td>
<td>0.86(s)</td>
<td>1.04</td>
<td>0.05</td>
<td>0.33</td>
<td></td>
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<tr>
<td>3-Hydroxybutyrate</td>
<td>Lipid</td>
<td>1.06(s)</td>
<td>1.26</td>
<td>0.08</td>
<td>-0.13</td>
<td>1.90(s)</td>
<td>4.70</td>
<td>&lt;0.001</td>
<td>-0.71</td>
<td></td>
<td>1.06(d)</td>
<td>1.45</td>
<td>&lt;0.01</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>3-Hydroxyisobutyrate</td>
<td>AA</td>
<td>2.54(s)</td>
<td>3.58</td>
<td>&lt;0.001</td>
<td>0.32</td>
<td>2.50(s)</td>
<td>0.82</td>
<td>0.04</td>
<td>0.08</td>
<td></td>
<td>1.82(d)</td>
<td>1.41</td>
<td>0.08</td>
<td>-0.23</td>
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<tr>
<td>Acetate</td>
<td>Carbohydrate</td>
<td>3.50(s)</td>
<td>1.65</td>
<td>0.55</td>
<td>-0.02</td>
<td>3.54(d)</td>
<td>2.23</td>
<td>0.04</td>
<td>0.07</td>
<td></td>
<td>7.62(s), 7.54(s)</td>
<td>0.82</td>
<td>0.01</td>
<td>-0.2</td>
<td></td>
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<tr>
<td>Glucose</td>
<td>Carbohydrate</td>
<td>2.34(s)</td>
<td>1.74</td>
<td>0.01</td>
<td>-0.19</td>
<td>2.34(s), 2.10(d)</td>
<td>1.24</td>
<td>0.02</td>
<td>-0.08</td>
<td></td>
<td>3.46(s), 3.54(d), 3.46(s), 3.26(d)</td>
<td>2.78</td>
<td>&lt;0.01</td>
<td>0.09</td>
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<tr>
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<td>AA</td>
<td>2.06(s)</td>
<td>3.32</td>
<td>0.02</td>
<td>-0.21</td>
<td>2.10(d), 2.06(s)</td>
<td>1.95</td>
<td>0.03</td>
<td>-0.09</td>
<td></td>
<td>2.42(d), 2.06(s)</td>
<td>2.57</td>
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<tr>
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<td>2.40</td>
<td>0.14</td>
<td>0.04</td>
<td>3.54(d)</td>
<td>2.23</td>
<td>0.04</td>
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<td>7.62(s), 7.54(s)</td>
<td>0.82</td>
<td>0.01</td>
<td>-0.2</td>
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<tr>
<td>Isoleucine</td>
<td>AA</td>
<td>3.62(s)</td>
<td>3.27</td>
<td>0.01</td>
<td>0.19</td>
<td>0.98(d)</td>
<td>1.59</td>
<td>0.24</td>
<td>0.06</td>
<td></td>
<td>3.62(s), 0.98(d)</td>
<td>2.54</td>
<td>&lt;0.01</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>AA</td>
<td>0.91(s)</td>
<td>2.51</td>
<td>0.2</td>
<td>-0.09</td>
<td>1.82(d)</td>
<td>1.03</td>
<td>&lt;0.01</td>
<td>-0.19</td>
<td></td>
<td>1.82(d)</td>
<td>1.41</td>
<td>0.08</td>
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<tr>
<td>Methionine</td>
<td>AA</td>
<td>3.34(s)</td>
<td>3.6</td>
<td>&lt;0.001</td>
<td>-0.28</td>
<td>3.34(s)</td>
<td>2.26</td>
<td>&lt;0.001</td>
<td>0.27</td>
<td></td>
<td>3.34(s)</td>
<td>2.87</td>
<td>&lt;0.001</td>
<td>-0.4</td>
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<tr>
<td>Phenylalanine</td>
<td>AA</td>
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<td>0.80</td>
<td>0.02</td>
<td>-0.11</td>
<td>2.14(s)</td>
<td>1.03</td>
<td>&lt;0.01</td>
<td>-0.19</td>
<td></td>
<td>7.34(s)</td>
<td>0.81</td>
<td>&lt;0.01</td>
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<td>Propionate</td>
<td>Carbohydrate</td>
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<td>1.53</td>
<td>0.11</td>
<td>-0.16</td>
<td>2.18(s)</td>
<td>1.01</td>
<td>&lt;0.001</td>
<td>-0.66</td>
<td></td>
<td>2.18(s), 1.06(d)</td>
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<td>&lt;0.01</td>
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<tr>
<td>Pyruvate</td>
<td>Carbohydrate</td>
<td>1.02(s)</td>
<td>1.22</td>
<td>0.46</td>
<td>-0.05</td>
<td>0.98(d)</td>
<td>1.59</td>
<td>0.24</td>
<td>0.06</td>
<td></td>
<td>3.58(s), 0.98(d)</td>
<td>2.54</td>
<td>0.01</td>
<td>0.27</td>
<td></td>
</tr>
</tbody>
</table>

1^s = singlet; d = doublet.
2^Variable importance in projection (VIP) was obtained from the partial least squares discriminant analysis.
3^P-values were calculated with Student’s t-test for unpaired data, whereas Student’s paired t-test was used for paired data.
4^Fold change (FC) was the ratio of mean of the former group/mean peak of the latter group.
In addition, 5 metabolites (citrate, creatinine, ethanol, histidine, and methionine) were associated with the cold responses only in Sanhe cattle. In the case of Holstein, 6 unique metabolites, including arginine, betaine, lysine, phenylalanine, 3-hydroxyisobutyrate, and 2-hydroxybutyrate, were the targeted features influenced by acute cold exposure. Of note, we found additional pathways of "arginine and proline metabo-
Figures 5. The differential metabolites and their pathways before and after acute cold exposure of Holstein cattle. (A) Scores plot of partial least squares discriminant analysis of metabolic profiling of Holstein before (HB) and after (HA) acute cold challenge. (B) Top 15 chemical shifts with higher variable importance in projection (VIP) scores. (C) The results of pathway analysis. (D) Key enzymes related to metabolism pathways. ACS = acyl-CoA synthetase; CS = citrate synthase; G-6-Pase = glucose-6-phosphatase. Student’s paired t-test was used to analyze the difference between HB and HA. Student’s paired t-test was used in the results presented in (D). Values are presented as mean ± SD.

Potential Biomarkers of Acute Cold Challenge in Cattle

Seven metabolites, including 3-hydroxybutyrate, acetate, glucose, methanol, propionate, glutamate, and glycine, were further identified as candidate biomarkers.
of acute cold challenge based on their VIP and P-values in foregoing analyses. Quantifications of those targeted plasma metabolites showed a discrepancy in abundance (Supplemental Table S5; https://doi.org/10.6084/m9.figshare.20010539.v1; Hu, 2022h). For instance, the metabolites with higher concentrations were acetate (10^{-2} mmol/L) and glucose (10^{-2} mmol/L), whereas methanol (10^{-3} mmol/L) and propionate (10^{-3} mmol/L) were detected with a lower level. Further, we observed substantial clustering between individuals in the acute cold exposure versus control groups (Figure 7A and 7B). Receiver operating characteristic curve analysis highlighted that the acetate (0.875), methanol (0.838), and propionate (0.787) had AUC values greater than 0.70 for Sanhe cattle, whereas the methanol (0.921), propionate (0.754), and glutamate (0.746) had greater

**Figure 6.** Summary of the identified differential metabolites and their metabolic pathways. (A) The Venn diagram of different metabolites in Sanhe and Holstein. (B) The Venn diagram of cold-associated pathways in Sanhe and Holstein. (C) Differential metabolites were observed in 2 breeds and their networks. Bold represents differential metabolites, red-colored symbols represent significantly higher metabolites in the latter group compared with the former group, white-colored symbols mean no difference. SB = Sanhe before acute cold challenge; SA = Sanhe after acute cold challenge; HB = Holstein before acute cold challenge; HA = Holstein after acute cold challenge; G-6-Pase = glucose-6-phosphatase; CS = citrate synthase; GS = glutamine synthetase; HIBADH = 3-hydroxyisobutyrate dehydrogenase.
AUC values for Holstein cattle (Figure 7C). Moreover, 6 metabolites significantly differed between the acute cold exposure group and the control group, which was consistent with the relative quantification (Figure 7D and 7E).

**DISCUSSION**

When exposed to cold temperatures, animals experience physiological, behavioral, and hormonal changes to maintain homeothermy (Verbeek et al., 2012; Bhimte et al., 2018). In cattle, these mechanisms require increasing energy for maintenance by up to 20% due to the changes in metabolic rate (Young, 1981). Consequently, fewer available nutrients for production will influence the performance in production efficiency. Previous studies in cattle have reported the effects of cold stress on physiology and behavior (Webster et al., 2008), endocrine (Bhimte et al., 2018), production (Brouček et al., 1991), as well as immune function (Kang et al., 2016). Under cold conditions, metabolic activity increases to generate more heat, which will partially result in synchronous changes in metabolism and metabolic pathways (Bhimte et al., 2018). The levels of metabolites can be regarded as the end products of the consequences of cellular regulatory responding to cold exposure.

More recently, the relationships between metabolites and physiological states, nutrient factors, genetic composition, and environmental effects have been investigated in numerous studies (Nicholson, 2006; Liao et al., 2018; Hao et al., 2020). In this context, this study was conducted to characterize metabolite profiles between Sanhe and Holstein cattle in thermoneutral conditions and evaluate the different mechanisms of metabolic regulation under acute cold exposure.

**Differences in Metabolic Profiles Between Sanhe and Holstein Cattle**

Significant differences were found in the plasma metabolome of Sanhe and Holstein cattle in thermoneutral conditions. Twenty differential metabolites were identified, in which the levels of glucose and pyruvate in Sanhe were lower than that of Holstein cattle, whereas citrate was higher in Sanhe cattle. The activity of CS, which is the first of a series of enzymes involved in the citric acid cycle (TCA cycle) and regulates the level of citrate (Roosterman and Cottrell, 2021), was significantly greater in Sanhe cattle. Moreover, G-6-Pase (glucose-6-phosphatase) plays an important role in maintaining the stability of blood glucose, and, therefore, its greater activity in Sanhe could indicate an increased usage of glucose for energy supply (Herling et al., 1999). These results suggest that glycolytic activity was greater in Sanhe than Holstein cattle.

The levels of AA and their derivatives, including arginine, glutamine, leucine, lysine, glutamate, valine, phenylalanine, and 3-hydroxyisobutyrate, were lower in the plasma of Sanhe cattle. Among them, arginine, glutamine, and glutamate participate in the TCA cycle by converting into 2-oxoguturate (Li et al., 2017). Leucine, lysine, and phenylalanine also relate with that pathway by producing acetyl-CoA which generates citrate by CS (Gu et al., 2018). Furthermore, 3-hydroxyisobutyrate is a downstream product of valine metabolism, and is finally converted to succinyl-CoA (Meyer et al., 2021). Its lower level is consistent with the higher activity of HIBADH (3-hydroxyisobutyrate dehydrogenase). These findings indicate that there was an extensive valine metabolism in Sanhe cattle. Therefore, in comparison with Holstein, Sanhe may have higher mobilization of AA.

The variations in genetic background between the 2 breeds lead to the observed differences in the levels of these metabolites and enzymes involved in glycolysis and AA metabolism. Sanhe is a dual-purpose cattle breed that is commonly described as both meat- and milk-producing animals (Xu et al., 2017; Hu et al., 2021). Holstein is a dairy cattle breed selected for high milk yield (Brito et al., 2021). Cattle breeds tend to differ based on feed intake, digestive efficiency, and weight gain (Archer and Bergh, 2000; Retallick et al., 2017). A previous study focusing on cattle growth traits found greater relative average daily gain in growing Simmental cattle, one of the most popular dual-purpose breeds worldwide, when compared with Holstein (Chen et al., 2013). In the present study, the Sanhe heifers weighed 392.3 ± 52.1 kg, whereas the Holstein weighed 376.3 ± 49.3 kg at similar ages. Although the BW was not significantly different between Sanhe and Holstein (P > 0.05), the requirements in nutrients could be considered to amplify throughout the biochemical process. Amino acids are required in growing animals, those abundant metabolites in plasma are the important source of protein biosynthesis in skeletal muscle (Geay, 1984; Leibel et al., 1995). The greater requirement for growth may be the reason why AA were lower in Sanhe cattle.

**Different Metabolic Responses to Acute Cold Challenge Between Sanhe and Holstein Cattle**

The core body temperature is one potential index to evaluate the effects of temperature exposure after a consequence of the physiological adaptations (Dikmen et al., 2012). Rectal temperature is commonly used to determine the core body temperature (Banerjee et al., 2015; Lee et al., 2016). In response to acute cold exposure, the changes in RT have been studied in multiple
Figure 7. Identification of potential biomarkers for acute cold challenge in cattle. (A) Hierarchical clustering of 7 metabolites between individuals in the cold exposure versus control group of Sanhe cattle. (B) Hierarchical clustering of 7 metabolites between individuals in the cold exposure versus control group of Holstein cattle. (C) The results of area under the curve (AUC) analysis for 7 metabolites. (D) The comparisons of acetate, methanol, and propionate before (SB) and after (SA) acute cold exposure of Sanhe. (E) The comparisons of methanol, propionate, and glutamate before (HB) and after (HA) acute cold exposure of Holstein. Student’s paired t-test was used in the results presented in (D) and (E). Values are presented as mean ± SD.
mammals, and a rising trend has been found because of the shift in circulating blood from the periphery to the core (Raven and Horvath, 1970; Doubek et al., 2003). In this study, significantly higher RT was observed when Holstein cattle were exposed to acute cold conditions; however, it did not occur in Sanhe cattle. These results suggest that there were different capabilities of cold regulation between the 2 breeds, and Sanhe had better performance on body temperature maintenance under acute cold exposure. In our previous studies, metabolic processes played important roles in the regulations of cold responses in cattle (Hu et al., 2021). To capture whether there were different mechanisms of metabolic regulation between Sanhe and Holstein, we further investigated the effect of cold exposure on their metabolome profiling. An important point to highlight is the time in which blood samples were collected, which can influence the final results. In this study, blood was collected soon after the acute cold challenge ended. However, blood sampling can be time consuming, and in some cases, it took up to 20 min. To evaluate the effect of sampling time, we compared the global metabolome profile of individual animals by classifying them into early-sampled and late-sampled. The PCA and PLS-DA plots indicated that was no clear clustering was found between the 2 groups (results not shown). Therefore, all individuals were included in further analyses. Eleven metabolites were altered in both Holstein and Sanhe cattle under cold conditions. This indicates that most regulators of metabolic pathways are similar in both breeds under acute cold exposure. Furthermore, metabolic changes associated with acute cold challenges had different patterns, fluctuations, as well as categories involved in the 2 cattle breeds. For example, the increased citrate and creatinine, as well as decreased ethanol, histidine, and methionine, were specially changed in Sanhe. A previous study in cold-stressed rats reported that the TCA cycle was accelerated with upregulated intermediates due to enhanced demand for energy, such as citrate (Wang et al., 2007). The citrate level significantly increased in cold-induced Sanhe cattle, which indicates that the TCA cycle may be rapidly triggered with a high concentration of baseline CS. Creatinine is the end product of creatine and creatine phosphate metabolism, which relates to energy balance when animals require energy in a short time (Brosnan and Brosnan, 2010). Therefore, the increased creatinine highlighted the important implications of creatine phosphate in Sanhe’s cold response. Additionally, a study found the generation of creatine used 42% methionine (Kashani et al., 2020). The reduced level of plasma methionine provided powerful evidence for the involvement of this investigated pathway in cold regulation. Histidine was downregulated in Sanhe cattle, which may be due to the degradation of this metabolite for producing glutamate (Brenchley, 1973).

For Holstein cattle, the levels of 2-hydroxybutyrate, 3-hydroxyisobutyrate, arginine, betaine, lysine, and phenylalanine were especially altered during cold exposure. However, only 2-hydroxybutyrate, which is formed as a product during the formation of 2-oxoglutarate (Nishiumi et al., 2012), had an increased level under acute cold challenge. The roles in competition with acetate metabolism and the relation between biotinidase deficiency have been previously demonstrated (Dutra-Filho et al., 1995). In addition, 2-oxoglutarate was suggested as an early marker in impaired glucose regulation, and the increase in lipid oxidation and oxidative stress (Gall et al., 2010). Our findings indicate that Holstein cattle might undergo a severe negative effect due to tissue hypoxia and oxidative stress under acute cold challenge. On the contrary, the other 5 compounds were significantly lower when Holstein animals were exposed to cold conditions. The drop level of 3-hydroxyisobutyrate may be the result of involvement in gluconeogenesis in the liver (Letto et al., 1986), and reveals the higher activity of valine metabolism. Similarly, lysine and phenylalanine are essential AA that can only be obtained from feed in cattle (Chandler and Polan, 1972). Their falling circulating levels reveal an increased use of those AA to counter the damage of acute cold challenge in Holstein.

**Potential Biomarkers for Acute Cold Challenge in Cattle**

Seven metabolites, including 3-hydroxybutyrate, acetate, glucose, glutamate, glycine, methanol, and propionate, were further selected with high power for discrimination of the control and cold exposure groups. Previous studies have identified biomarker metabolites for heat-stressed dairy cows (Tian et al., 2015), beef cattle (Liao et al., 2018), pigs (Dou et al., 2017), and broilers (Lu et al., 2018). However, there are limited biomarker compounds for livestock under cold conditions, especially for cattle. Therefore, the metabolites discovered in this study provide important insights into potential biomarkers metabolites for acute cold challenges in cattle.

Good examples are propionate and methanol, which had high AUC values (>0.70) in both Sanhe and Holstein. Generally, biomarkers with AUC >0.70 are considered as being fairly sensitive and specific in diagnostic power (Dervishi et al., 2017). Propionate, with AUC values of 0.787 in Sanhe and 0.754 in Holstein, is a VFA from the metabolism of microorganisms in the rumen (Wolin, 1960), which is absorbed into the blood by epithelial cells (Masson and Phillipson, 1951). Wiltrout
and Satter (1972) have reported that propionate could be considered the major substrate for gluconeogenesis to supply energy. In the present study, the concentration of propionate decreased when both breeds were exposed to cold temperature, which suggests that propionate may act as a key source in the process of gluconeogenesis at the beginning of cold challenge. Also, methanol is the production of microbial fermentation for pectin in the rumen. It can be used as a substrate for methanogenic bacteria to produce methane, or it will pass through the ruminal epithelium into the blood circulation (Liu and Whitman, 2008). The values of AUC were 0.839 and 0.921 in Sanhe and Holstein cattle, respectively, illustrating the significant associations between the level of methanol and acute cold exposure. Interestingly, the adverse changing trends of methanol under cold conditions were identified between Sanhe and Holstein. This result may be due to the different characteristics in the microorganism’s reaction under acute cold conditions. This connection requires experimental validation based on metagenome sequencing.

**Implications and Next Steps**

Our results have pointed out the different metabolic changes of 2 important breeds under short-term cold exposure, and identified potential metabolic biomarkers related to acute cold challenge in cattle. Based on these findings, a better understanding of the biological mechanisms of cold resilience was obtained, and prospective metabolic phenotypes corresponding to acute cold responses will be helpful to optimize breeding strategies. Additional research is needed to test the metabolic regulations of different cold intensities and durations in cattle.

**CONCLUSIONS**

Blood metabolic profiles from Sanhe and Holstein heifers indicated breed-specific characteristics associated with metabolic regulation and responses to acute cold challenge. Under thermoneutral conditions, 20 differential metabolites were identified between 2 breeds, and involved in glycolysis and AA metabolism. Lower levels of related metabolites were found in Sanhe cattle, indicating the greater glycolytic activity and mobilization of AA. In response to the acute cold challenge, 16 and 17 differential metabolites were investigated in the comparisons of Sanhe before versus after cold exposure and Holstein before versus Holstein after cold exposure, respectively. Among them, 11 metabolites were altered in both breeds, and highlighted the fact that studied animals responded to acute cold challenge by regulating the metabolism of VFA and AA for gluconeogenesis, with an increased glucose level. Breed-specific metabolites demonstrated that Sanhe cattle mobilized greater TCA cycle activity, and creatine and creatine phosphate metabolism, whereas Holstein cattle tended to employ the metabolism of AA to supply energy. However, unchanged body temperature was only observed in Sanhe cattle, indicating that this local breed has higher efficiency of thermoregulation under acute cold challenge. Additionally, propionate and methanol are expected to be potential biomarkers for evaluating acute cold challenge in cattle.

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Hu et al.: METABOLOME ANALYSES OF SANHE AND HOLSTEIN