

## <sup>1</sup>H NMR-based Plasma Metabolic Profiling of Dairy Cows with Type I and Type II Ketosis

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### Abstract

This study identified differences in plasma metabolites among three groups of dairy cows: type I ketotic (K1), type II ketotic (K2), and healthy control cows (C). 50 cows with two or three parities were selected at 7–28 days postpartum. Cows were classified as type I ketotic (K1, 20 cows), type II ketotic (K2, 20 cows), or healthy control cows (C, 10 cows). Plasma metabolomic profiles were analyzed by <sup>1</sup>H-nuclear magnetic resonance technology (<sup>1</sup>H NMR). The data were processed by principal component analysis and orthogonal partial least-squares discriminant analysis (OPLS-DA). Results-The results revealed that OPLS-DA was more effective at distinguishing amongst the three groups. Additionally, there were seven different metabolites between K2 and C, 19 different metabolites between K1 and C, and 24 different metabolites between K1 and K2. Therefore, the combination of <sup>1</sup>H-NMR and multivariate statistical analyses can effectively distinguish the differential metabolites among the K1, K2, and C groups, thereby providing important information on the pathogenesis, early diagnosis, and prevention of type I and type II ketosis in dairy cows.

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### Keywords

<sup>1</sup>H NMR; Metabolomics; Type I Ketosis; Type II Ketosis; Multiple analysis

### Introduction

Ketosis is a metabolic disease that affects dairy cows during transition periods [1]. In general, ketosis is closely related to negative energy balance (NEB), poor appetite, and high milk production during early lactation, which may contribute to inadequate energy intakes [2]. There are two types of ketosis: clinical ketosis and subclinical ketosis, based on the clinical symptoms, e.g., anorexia, depression, decreased milk production, constipation, drowsiness or nervousness [3], and plasma 3-hydroxybutyrate acid levels (BHBA, >1.20 mmol/L) [4]. However, Holtenius et al. [5] reported that ketosis in dairy cattle is divided into types I and II based on the disease characteristics and clinical features. Type I ketosis, which is mainly attributed to the absence of gluconeogenic precursors, is also termed secondary ketosis, alimentary ketosis, starvation ketosis, and special nutritional deficiency ketosis. Type I ketosis generally occurs 3–6 weeks postpartum and is

characterized by hypoglycemia, hypoinsulinemia, and high fatty acid and ketone levels. Type II ketosis, which is associated with hepatic fat deposition, occurs 5–15 days postpartum. Type II ketosis is characterized by hyperglycemia or normal glucose levels, hyperinsulinemia, and high non-essential fatty acid (NEFA) levels. Cows with type II ketosis have insulin resistance and glucose tolerance [6]. Metabolomics is an emerging and important technology in systems biology, which can be implemented in biological samples for comprehensive qualitative and quantitative analyses [7]. Currently, metabolomics comprise nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and metabolite chips [8]. <sup>1</sup>H-nuclear magnetic resonance technology (<sup>1</sup>H-NMR) is a non-invasive technology that allows sample testing with good objectivity and reproducibility, no laborious pre-treatment steps, high throughput, and low costs compared to GC-MS or LC-MS [9]. Furthermore, <sup>1</sup>H-NMR allows a complete detection of most compounds in the sample [10]. The discovery of disease biomarkers by <sup>1</sup>H-NMR coupled to multivariate statistical analyses will provide novel ideas regarding disease pathogenesis and prevention strategies. Metabolomics has not only been used in metabolic profiles and pathogenesis of human diseases (e.g., liver cirrhosis, bladder cancer, diabetes, and lupus erythematosus) [11-14], but also in the description of pathological diseases, discovery of novel biomarkers, and elucidation of metabolic regulatory pathways. Studies have focused on the metabolomics of type I ketosis [15-24]; however, there are no studies on the metabolomics of type II ketosis. In this study, we used

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Received October 12, 2014; Accepted January 05, 2015; Published January 12, 2015

Citation: Xu C, Li Y, Xia C, Zhang HY, Sun LW (2015) <sup>1</sup>H NMR-based Plasma Metabolic Profiling of Dairy Cows with Type I and Type II Ketosis. Pharm Anal Acta 6: 328. doi:10.4172/2153-2435.1000328

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<sup>1</sup>H-NMR technology to compare the metabolomics of type I and type II ketosis. The objective of this study was to understand the clinical pathology and pathogenesis of type II ketosis.

## Materials and Methods

### Experimental animals

Animals were selected from an intensive dairy farm in Heilongjiang, China. In this study, 50 cows with two or three parities were selected at 7–28 days postpartum. Cows were classified as type I ketotic (K1, 20 cows), type II ketotic (K2, 20 cows), or healthy control cows (C, 10 cows), based on the glucose (Glc) and 3-hydroxybutyrate acid (BHBA) levels and the clinical signs. Type I ketosis was defined as having plasma BHBA >1.20 mmol/L, plasma Glc <2.50 mmol/L, and plasma NEFA >0.50 mmol/L. Type II ketosis was defined as having plasma BHBA >1.20 mmol/L, Glc >2.80 mmol/L, and plasma NEFA >0.50 mmol/L. Healthy controls were those with plasma BHBA <1.00 mmol/L, plasma Glc >3.75 mmol/L, and plasma NEFA <0.40 mmol/L. All the animals were fed a total mixed ration (TMR) at pre-partum, which consisted of 8.5 kg concentrated feed, 18.5 kg silage maize, 4 kg hay, and 350 g fat. The composition was 55.60% DM (dry matter), 16% crude protein, 1.75 mcal/DM NEL (net energy for lactation), 5.60% fat, 39.10% NDF (neutral detergent fiber), 20.30% ADF (acid detergent fiber), 180 g Ca, and 116 g P.

### Sample collection and blood parameter analyses

Blood samples (10 ml) from all three groups were collected from the caudal vein, mixed with heparin, and centrifuged at 1,400 g for 10 min at room temperature. The resulting supernatant was transferred to Eppendorf tubes (1 ml plasma/tube) and stored at -80°C. Both clinical and blood parameters were measured. The clinical parameters included age, parity, body condition score, and milk yield (MY). The blood parameters included Glc (by the glucose oxidase method, mmol/L), BHBA (by the enzyme dynamics method, mmol/L), NEFA (by ELISA, mmol/L), triglycerides (TGs, by the enzyme reagent method, mmol/L), aspartate aminotransferase (AST, by the velocity method, U/L), and insulin (Ins, by ELISA, mIU/L). The commercial kits for measuring Glc, TG, and AST were purchased from Changchun Huili Biotechnology Co., Ltd. (Changchun, China); the BHBA commercial kit was purchased from Randox Laboratories Ltd. (Belfast, UK). All the other kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The blood parameters were measured with an automatic biochemical analyzer (Roche, Germany) at the College of Animal Science and Veterinary Medicine of Heilongjiang Bayi Agricultural University.

### Sample preparation and <sup>1</sup>H-NMR spectroscopy

In this experiment, 300 µl of thawed serum sample was mixed with 150 µl buffer solution (0.2 mol/L Na<sub>2</sub>HPO<sub>4</sub> and 0.2 mol/L NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4) and 150 µl sodium 3-trimethylsilyl-(2, 2, 3, 3-D4) propionate (TSP, 1 mg/ml, Sigma-Aldrich) in D<sub>2</sub>O. Aliquots (550 µl) were transferred to 5-mm NMR tubes. <sup>1</sup>H-NMR spectra were obtained in a 500 MHz Bruker AV500 spectrometer at 300 K. The water-suppressed Carr-Purcell-Meibom-Gill (CPMG) spin-echo pulse sequence (RD-90°-(τ-180°-τ)n-ACQ) with a total spin-echo delay (2nτ) of 40 ms was used to suppress broad signals from macromolecules (i.e., proteins and lipoproteins), where signals of micromolecules are obtained. Prior to Fourier transformation, an exponential line broadening function of 0.5 Hz was applied to the FID. All plasma <sup>1</sup>H-NMR spectra were manually phased, baseline-corrected, and referenced to TSP (CH<sub>3</sub>, δ0.0), using Bruker Topspin 3.0 software (Bruker GmbH, Karlsruhe, Germany).

## Data Processing and Statistical Analyses

### The analysis of clinical and blood parameters

Both clinical and blood parameters were analyzed by one-way analysis of variance (ANOVA). Statistical significance was set at *P* < 0.05.

### <sup>1</sup>H-NMR spectra

<sup>1</sup>H-NMR spectra were subjected to Fourier transformation, phase adjustment, baseline-correction, and calibration using MestReNova software (V7.0). To improve the signal-to-noise ratio, all spectra were multiplied by a 1.0-Hz exponential function prior to Fourier transformation. <sup>1</sup>H-NMR spectra were referenced to an internal lactic acid CH<sub>3</sub> resonance at 1.33 ppm. <sup>1</sup>H-NMR spectra were segmented into consecutive non-overlapping regions of 0.002-ppm chemical shift “bins” between 0.5 and 9.0 ppm; the residual water peak at 4.18-6.70 ppm was removed from the data.

### Multivariate statistical analyses

The normalized data were analyzed by multivariate analysis and pattern recognition methods with SIMCA-P<sup>+</sup> software (V11.0, Umetrics AB, Umea, Sweden). First, <sup>1</sup>H-NMR spectra was analyzed by principal component analysis (PCA), where the scale data conversion with mean center scaling reflects the total metabolic differences among the three groups and the degree of variability within each group [16]. Following PCA, the three groups of plasma metabolites were analyzed by orthogonal partial least-squares discriminant analysis (OPLS-DA). OPLS-DA is based on unit variance scaling, which can emphasize any differences among groups. R<sub>2</sub>X and Q<sup>2</sup> are models of quality evaluation: R<sup>2</sup>X describes the degree of optimization of the analytical model and Q<sup>2</sup> describes the cumulative prediction of the model. If R<sup>2</sup> and Q<sup>2</sup> are >0.4, the model is considered to be reliable [17]. We summarized the statistical significance of the metabolites by OPLS-DA and correlation coefficients. The product from the loading values for each variable and the square root of the standard deviation were retrospectively transformed. Differences in metabolites among the groups were determined by the critical value of the corresponding correlation coefficients.

## Results

### Clinical information

The clinical data from the K1, K2, and C groups are in Table 1. The differences among the three groups in post-partum day, MY, Ins, AST, and TG were not significant (*P* > 0.05). However, body condition scores, Glc, BHBA, and NEFA were significantly different among the three groups (*P* < 0.05).

### <sup>1</sup>H-NMR Spectroscopy of plasma samples

The representative 600 MHz CPMG <sup>1</sup>H-NMR spectra (δ0.5–5.5 and δ5.5–9.0) of samples from K1, K2, and C groups Figure 1. The δ5.5–9.0 region (dashed box) was magnified 10 times relative to the δ0.5–5.5 region for clarity. The samples K<sub>1-15</sub>, K<sub>1-16</sub>, K<sub>2-8</sub>, K<sub>2-16</sub>, and C<sub>8</sub> were removed to achieve a better quality of spectrum and a better suppression effect of the water peak. The data were subsequently analyzed using multivariate statistics (PCA and OPLS-DA).

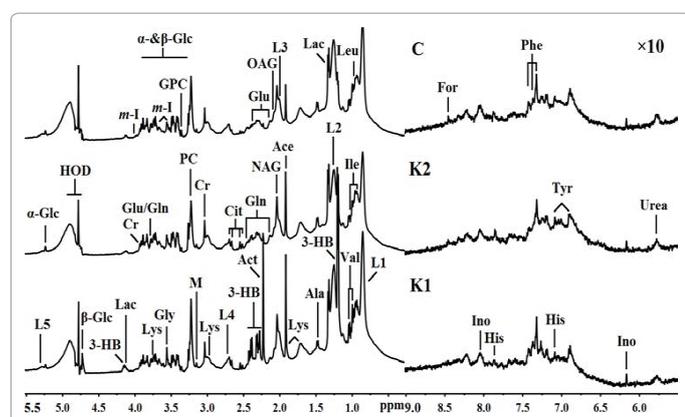
### Multivariate statistical analyses

The resulting scores plot (R<sup>2</sup>X= 68.0%, Q<sup>2</sup>= 0.549) of PCA is shown in Figure 2. PCA results revealed that the three groups were scattered

Parameter	K1 group	K2 group	C group
Milk yield (kg/d)	28.99 ± 7.37	30.82 ± 8.19	34.07 ± 6.70
Postpartum day (d)	16.15 ± 4.50	15.20 ± 5.01	16.30 ± 2.95
Body condition score	3.07 ± 0.38 <sup>ab</sup>	3.17 ± 0.28 <sup>a</sup>	2.80 ± 0.33 <sup>b</sup>
Glc (mmol/L)	2.03 ± 0.33 <sup>A</sup>	3.31 ± 0.57	3.02 ± 0.30
BHBA (mmol/L)	3.35 ± 1.48 <sup>A</sup>	1.85 ± 0.67 <sup>B</sup>	0.76 ± 0.34 <sup>C</sup>
NEFA (mmol/L)	1.42 ± 0.65 <sup>A</sup>	0.98 ± 0.32 <sup>B</sup>	0.35 ± 0.12 <sup>C</sup>
Ins (mIU/L)	6.60 ± 1.25	6.90 ± 1.08	7.07 ± 1.07
AST (U/L)	28.53 ± 19.41	52.24 ± 51.42	49.15 ± 31.89
TG (mmol/L)	0.09 ± 0.02	0.09 ± 0.01	0.09 ± 0.02

Different uppercase and lowercase letters within a row represent significant differences at  $P < 0.01$  and  $P < 0.05$ , respectively.

**Table 1:** Clinical data and biochemical parameters in three groups of dairy cows



**Figure 1:** Representative 600 MHz CPMG <sup>1</sup>H-NMR spectra ( $\delta$ 0.5–5.5 and  $\delta$ 5.5–9.0) of samples obtained from groups K1, K2, and C. The  $\delta$ 5.5–9.0 region was magnified 10 times relative to the corresponding  $\delta$ 0.5–5.5 region for the purpose of clarity.

3-HB: 3-Hydroxybutyrate; Ace: Acetate; Act: Acetone; Ala: Alanine; Cit: Citrate; Cr: Creatine; For: Formate; Gln: Glutamine; Glu: Glutamate; Gly: Glycine; GPC: Glycerolphosphocholine; His: Histidine; Ile: Isoleucine; Ino: Inosine; L1: LDL&VLDL,  $\text{CH}_2(\text{CH}_2)_n$ ; L2: LDL&VLDL,  $\text{CH}_3(\text{CH}_2)_n$ ; L3: Lipid,  $-\text{CH}_2-\text{CH}=\text{CH}-$ ; L4: Lipid,  $-\text{CH}=\text{CH}-\text{CH}=\text{CH}-$ ; L5: Lipid,  $-\text{CH}=\text{CH}-$ ; Lac: Lactate; Leu: Leucine; Lys: Lysine; *m*-I: *myo*-Inositol; NAG: N-acetyl glycoprotein signals; OAG: O-acetyl glycoprotein signals; PC: Phosphocholine; Phe: Phenylalanine; Tyr: Tyrosine; Val: Valine;  $\alpha$ -Glc:  $\alpha$ -Glucose;  $\beta$ -Glc:  $\beta$ -Glucose.

in different areas; therefore, K1, K2, and C were significantly different. However, the K2 samples were distributed in the regions of K1 and C, which indicates that the composition and concentration of some variables were similar among the group samples. Two outliers from K1 were spotted outside the 95% significance region of Hotellings T2 ellipse. The data were analyzed by OPLS-DA to reduce errors within the group and eliminate random errors. K1 and K2 were discriminated with  $R^2X = 66.2\%$  and  $Q^2 = 0.473$ ; K1 and C were discriminated with  $R^2X = 71.3\%$  and  $Q^2 = 0.507$ ; and K2 and C were discriminated with  $R^2X = 50.9\%$  and  $Q^2 = 0.333$ . In the OPLS-DA score plots Figure 3, there were significant differences in metabolic patterns between any two groups with clear differences in the metabolic spectrum.

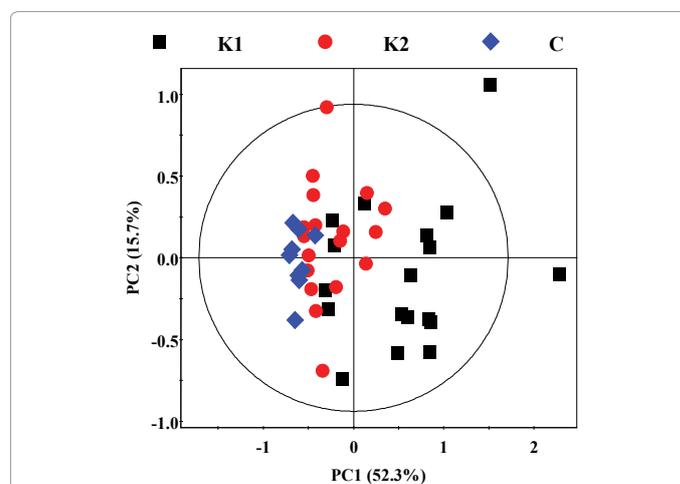
### Metabolites

The results revealed that there were significant differences in the plasma metabolites among the three groups Table 2. Compared with C, K1 had higher levels of BHBA and acetone and lower levels of tyrosine, phenylalanine, lysine, histidine, alanine, creatine, *myo*-Inositol,  $\beta$ -Glc, glutamine, glutamate, citrate,  $\alpha$ -Glc, formate, glycine, O-acetyl glycoprotein (OAG), and phosphocholine. K2 had higher plasma levels of BHBA, acetone, and lactate and lower levels of alanine,

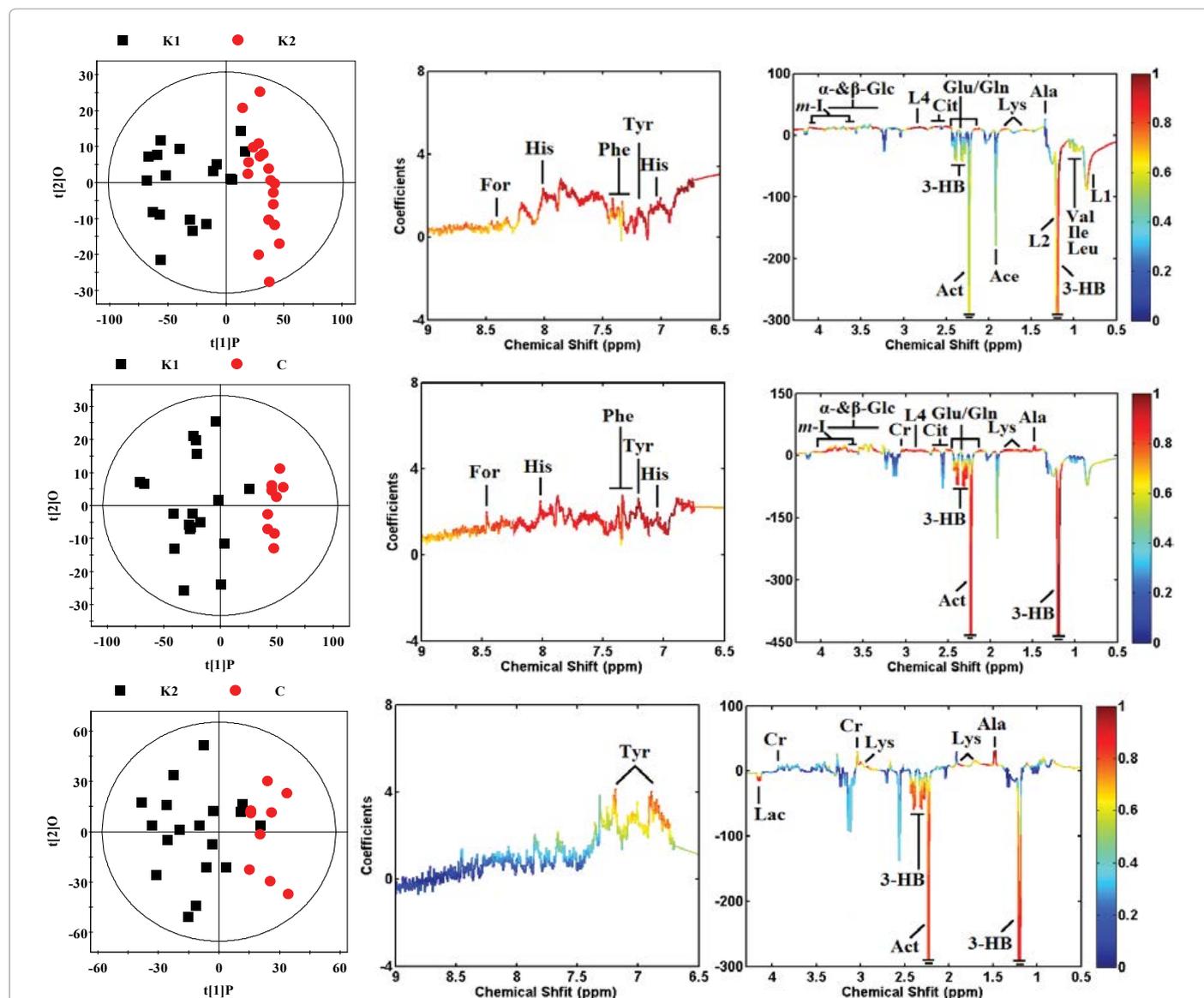
lysine, tyrosine, and creatine. However, compared with K2, K1 had higher levels of BHBA, low density lipoprotein (LDL), very low density lipoprotein (VLDL), isoleucine, valine, acetone, leucine, and acetate and lower levels of citrate, tyrosine, histidine, creatine, glutamine,  $\beta$ -Glc, phenylalanine, glutamate,  $\alpha$ -Glc, lysine, formate, glycine, phosphocholine, and OAG. Therefore, there were different metabolic pathways among type I ketosis, type II ketosis, and control groups.

### Discussion

This study assessed the metabolic characteristics of type I ketosis and type II ketosis using <sup>1</sup>H-NMR technology. The metabolism of carbohydrates, lipids, and proteins in dairy cows is closely associated with energy supply [18]. Macronutrient metabolism requires a dynamic balance (i.e., anabolism and catabolism), to maintain life activities; otherwise, diseases occur [19]. According to the results, there were different peaks in the <sup>1</sup>H-NMR spectra among the three groups; these peaks corresponded to plasma metabolites (e.g. amino acids, glucose, and lipoprotein), which may be involved in glucose, lipid, and amino acid metabolism. Type I ketosis is a metabolic disorder that results from the absence of gluconeogenic precursor. Plasma metabolites in K1 either increased or decreased Table 2. Researchers have reported that [20] the demand for glucose by early lactating cows is closely related to cow ketosis in terms of glucose metabolism. NEB may occur within a few days after parturition because the energy demand increases rapidly during early lactation, resulting in glucose imbalance, decreased blood glucose, and increased ketone synthesis, leading to type I ketosis. The tricarboxylic acid (TCA) cycle is an important metabolic cycle; any disturbance in the TCA cycle affects other metabolic pathways. Citric acid, which is a TCA intermediate, is formed from acetyl CoA and oxaloacetic acid in a reaction catalyzed by citrate synthase [21]. However, in this study, plasma citric acid decreased in the K1 group, suggesting that TCA was blocked. In terms of lipid metabolism, because the energy demand is higher than the energy consumed during early lactation, NEB occurs and blood glucose levels decrease [22]. Additionally, NEB stimulates fat mobilization, resulting in the conversion of NEFAs into ketone bodies (i.e., acetone, acetoacetate, and BHBA). If NEB cannot be effectively alleviated, ketone body production will dramatically increase. When ketogenesis is greater than the ability of the body organs to utilize the resulting ketone bodies, ketosis will inevitably occur [23]. Therefore, excessive fat mobilization



**Figure 2:** PCA scores plot based on <sup>1</sup>H CPMG NMR spectra of plasma samples obtained from three groups. Type I ketosis (K1): black square; Type II ketosis (K2): red circle; control (C): blue diamond.



**Figure 3:** OPLS-DA score plots (left panel) derived from <sup>1</sup>H NMR spectra of serum samples and corresponding coefficient loading plots (right panel) obtained from the different groups. The color map shows the significance of variations in metabolites between the two classes. Peaks in the positive direction indicate that metabolites are more abundant in the groups in the positive direction of first principal component. Consequently, metabolites that are more abundant in the groups in the negative direction of first primary component are presented as peaks in the negative direction.

and high energy demands play crucial roles in the pathogenesis of ketosis [24]. Our results revealed that BHBA and acetone were higher in K1, consistent with the findings of other researchers [25]. In amino acid metabolism, the anabolism and catabolism of proteins need to be in dynamic balance to maintain normal physiological functions. Protein metabolic abnormalities result from abnormal anabolism, catabolism, or absorption. When NEB is severe, glucogenic amino acids (e.g., alanine, glycine, glutamic acid, glutamine, histidine, phenylalanine, and tyrosine) may play a role in regulating glucose metabolism [26]. Our results revealed that some glucogenic amino acids decreased in K1, suggesting that they were utilized to meet energy demands of dairy cows during early lactation. The reduction of histidine, phenylalanine, and tyrosine, may reduce neurotransmitter levels such as histamine and dopamine and contribute to endocrine disorders [27]. The etiology of type II ketosis is partly or completely attributed to a deficiency in insulin, a lack of insulin receptors, or poor insulin receptor sensitivity,

which may result in poor glucose utilization, high blood glucose levels, and disorders in lipid and amino acid metabolism [28]. Type II ketosis, which usually occurs in cows during the dry period, is similar to non-insulin-dependent diabetes (type II diabetes). The low insulin sensitivity may prompt fat mobilization and glucose synthesis. However, insulin resistance decreases lipid catabolism and enhances ketone body production, thereby contributing to type II ketosis. Normal glucose metabolism is essential to maintain energy metabolism in healthy dairy cows. Pyruvate molecules resulting from glycogenolysis are completely degraded in mitochondria for energy production [29]. Skeletal muscle cells oxidize glucose via glycolysis and produce energy. Lactic acid from anaerobic glycolysis may be converted into glucose by hepatic gluconeogenesis [30]. However, lactate significantly increased in K2 compared with K1; therefore, more glycolysis occurred in K2 to meet the energy demands of type II ketosis. There were different metabolites in K1 and K2: plasma BHBA, acetic acid, and acetone levels were lower

	Metabolites	r <sup>a</sup>		
		K1-K2	K1-C	K2-C
1	3-Hydroxybutyrate: 1.20(d), 2.31(dd), 2.41(dd), 4.16(m)	-0.861	-0.949	-0.780
2	Acetate :1.92(s)	-0.524	-	-
3	Acetone : 2.23(s)	-0.626	-0.865	-0.770
4	Alanine : 1.48(d)	0.583	0.883	0.905
5	Citrate : 2.53(d), 2.68(d)	0.931	0.843	-
6	Creatine : 3.04(s), 3.93(s)	-	0.870	0.762
7	Formate : 8.46(s)	0.690	0.799	-
8	Glutamate: 2.14(m), 2.35(m), 3.78(t)	0.810	0.846	-
9	Glutamine : 2.14(m), 2.45(m), 3.78(t)	0.882	0.846	-
10	Glycine : 3.56(s)	0.681	0.739	-
11	Histidine : 7.09(s), 7.87(s)	0.906	0.901	-
12	Isoleucine : 0.94(t), 1.01(d)	-0.673	-	-
13	L1: CH <sub>3</sub> -(CH <sub>2</sub> ) <sub>n</sub> -(LDL&VLDL): 0.87(br)	-0.816	-	-
14	L2: CH <sub>3</sub> -(CH <sub>2</sub> ) <sub>n</sub> -(LDL&VLDL): 1.27(br)	-0.629	-	-
15	L4: Lipid, =CH-CH <sub>2</sub> -CH= : 2.75(br)	0.924	0.817	-
16	Lactate : 1.33(d), 4.12(q)	-	-	-0.681
17	Leucine : 0.96(d)	-0.624	-	-
18	Lysine : 1.71(m), 1.90(m), 3.01(m), 3.76(t)	0.766	0.902	0.843
19	myo-Inositol : 3.59(dd), 3.66(dd), 4.06(m)	0.887	0.869	-
20	OAG: O-acetyl glycoprotein signals: 2.08(s)	0.539	0.725	-
21	Phenylalanine : 7.32(m), 7.37(m), 7.42(m)	0.841	0.906	-
22	Phosphocholine : 3.22(s)	0.608	0.695	-
23	Tyrosine : 6.90(d), 7.19(d)	0.908	0.943	0.736
24	Valine : 0.99(d), 1.04(d)	-0.661	-	-
25	α-Glucose: 3.42(t), 3.54(dd), 3.71(t), 3.73(m), 3.84(m), 5.22(d)	0.783	0.842	-
26	β-Glucose: 3.24vc(dd), 3.41(t), 3.46(m), 3.49(t), 3.90(dd), 4.65(d)	0.849	0.854	-

<sup>a</sup>Correlation coefficients: positive and negative signs indicate positive and negative correlation in the concentrations, respectively. The correlation coefficient  $|r| > 0.632$  or  $0.444$  was used as the cutoff value for the statistical significance based on the discrimination significance at  $P = 0.05$  and  $df$  (degree of freedom) = 8 or 18; “-” means that the correlation coefficient  $|r|$  is less than the cutoff value. <sup>b</sup>Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet.

**Table 2:** OPLS-DA coefficients derived from the NMR data of metabolites present in serum samples obtained from different groups.

in K2 than in K1. Acetic acid, which is a breakdown product of NEFAs, may be activated by acetyl CoA and transformed into ketone bodies [31]. However, insulin can promote fat synthesis and inhibit ketone body production. The levels of α-glucose and β-glucose were higher in K2 than in K1, probably due to the low insulin sensitivity of K2. Compared with K1, K2 had higher plasma VLDL/LDL. VLDL is composed of TGs, apolipoproteins, phospholipids, and cholesterol. Endogenous TGs can be synthesized in the liver and transported to other organs in the form of VLDL particles [32]. Endogenous cholesterol is transported by LDL particles. When cholesterol is transported back to the liver, bile acid can be synthesized [33]. When VLDL and LDL increase, plasma TGs and cholesterol in plasma similarly increase. NEB may be improved by fat mobilization; VLDL and LDL levels increase in type I ketosis. Insulin is a key hormone that regulates fat synthesis by inducing the synthesis of acetyl CoA carboxylase, ATP-citrate lyase, and fatty acid synthase [34]. Therefore, insulin resistance contributes to high lipid levels and low VLDL and LDL levels.

Amino acids and proteins are important components of cell structure. The structural integrity of insulin receptors affects insulin function and amino acid and protein metabolism. Insulin promotes glucose utilization and increases the synthesis of protein and fat [35]. In this experiment, glucose utilization was more affected in K2 than in K1 because there were considerable less glucogenic amino acids in K2 than in K1. Isoleucine, leucine, valine, and other branched-chain amino acids have similar metabolic processes [36]. Succinyl CoA and acetyl CoA participate in glucose metabolism and provide energy [37]. The reduction of glucogenic amino acids in K2 may affect the

TCA cycle and glycolysis due to the absence of TCA and glycolytic intermediates. Branched-chain amino acids are important amino acids during long periods of high energy requirements. Branched-chain amino acid may inhibit glycogenolysis in liver and muscle and enhance the alanine-glucose and lactic acid-glucose cycles to support gluconeogenesis. These processes are important energy sources during long periods of high energy requirements [38]. When glucose utilization is affected as in K2, the energy provided by the branched-chain amino acids can meet the high energy requirements. Therefore, branched-chain amino acids represent an approach to prevent and treat type II ketosis. Lysine is a ketogenic amino acid, which may be utilized to generate ketone bodies for energy during lactation. The carnitine precursor synthesized from lysine can participate in fat metabolism. Additionally, lysine can improve the anti-stress capacity [39]. Lysine content in plasma significantly decreased in K2 and K1, which may be related to an inadequate supply of feed. Therefore, ketosis in dairy cows can be prevented by feeds containing lysine. Our results revealed that creatine levels in the C group were higher than in K2 or K1 groups. Creatine is an important energy compound, which is synthesized from arginine, glycine, and methionine. Creatine plays roles in energy supply, strength, muscle growth, and fatigue recovery [40]. Creatine enters cells by a creatine transporter, which can be hindered by insulin [41]. However, creatine can quickly provide energy and relieve NEB [42]. Therefore, creatine as an energy buffer was utilized under NEB in dairy cows with ketosis. Serum creatine may be used as an indicator of NEB severity. Plasma phosphorylcholine levels were lower in the K1 group than in the K2 or C groups. Phosphorylcholine is a component

of cell membranes; an increase in serum phosphorylcholine levels is indicative of cell death due to inflammation or necrosis [43]. Choline can reduce fat in the liver by promoting the formation of phospholipids and improve fatty acid utilization in the liver [44]. Therefore, lipid levels increase as choline decreases; choline can be considered an important risk factor of type I ketosis. Plasma inositol levels in the K2 group were higher than in the C group. Inositol is essential in the regulation of cell growth and function. During fat metabolism, lecithin can be synthesized from choline [45]. In this study, the plasma levels of inositol decreased due to fat degradation in type I ketosis. Plasma formate levels significantly decreased in K1. Formate provides a carbon source for nucleic acid biosynthesis. Inflammatory diseases often occur as a result of excessive formate consumption [46]. Even though cows with ketosis are more vulnerable to mastitis and endometritis [47], it is crucial to understand the relationship between formate and ketosis in dairy cows.

## Conclusions

Plasma metabolites in type I ketotic, type II ketotic, and healthy cows were assessed by <sup>1</sup>H NMR and bioinformatics analysis. The results of this study provide more insight regarding the pathogenesis, diagnosis, and prevention of type I and type II ketosis in dairy cows.

## Acknowledgements

We are grateful for the financial support provided by the Chinese National Science and the Technology Department Project (2013BAD21B01-2, 2012BAD12B05-2 and 2012BAD12B03-2).

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