¹H-Nuclear magnetic resonance-based plasma metabolic profiling of dairy cows with clinical and subclinical ketosis

L. W. Sun,* H. Y. Zhang,*¹ L. Wu,* S. Shu,* C. Xia,*† C. Xu,* and J. S. Zheng*

*Department of Clinical Veterinary Medicine, Animal Science and Technology College, Heilongjiang August First Land Reclamation University, Daqing 163319, China

†Synergetic Innovation Center of Food Safety and Nutrition, Northeast Agricultural University, Harbin 150030, China

ABSTRACT

The purpose of this study was to assess the metabolic profile of plasma samples from cows with clinical and subclinical ketosis. According to clinical signs and 3-hydroxybutyrate plasma levels, 81 multiparous Holstein cows were selected from a dairy farm 7 to 21 d after calving. The cows were divided into 3 groups: cows with clinical ketosis, cows with subclinical ketosis, and healthy control cows. ¹H-Nuclear magnetic resonance-based metabolomics was used to assess the plasma metabolic profiles of the 3 groups. The data were analyzed by principal component analysis, partial least squares discriminant analysis, and orthogonal partial least-squares discriminant analysis. The differences in metabolites among the 3 groups were assessed. The orthogonal partial least-squares discriminant analysis model differentiated the 3 groups of plasma samples. The model predicted clinical ketosis with a sensitivity of 100% and a specificity of 100%. In the case of subclinical ketosis, the model had a sensitivity of 97.0% and specificity of 95.7%. Twenty-five metabolites, including acetoacetate, acetone, lactate, glucose, choline, glutamic acid, and glutamine, were different among the 3 groups. Among the 25 metabolites, 4 were upregulated, 7 were downregulated, and 14 were both upregulated and downregulated. The results indicated that plasma ¹H-nuclear magnetic resonance-based metabolomics, coupled with pattern recognition analytical methods, not only has the sensitivity and specificity to distinguish cows with clinical and subclinical ketosis from healthy controls, but also has the potential to be developed into a clinically useful diagnostic tool that could contribute to a further understanding of the disease mechanisms.

Key words: ¹H-nuclear magnetic resonance, clinical ketosis, subclinical ketosis, metabolic profiling

INTRODUCTION

Ketosis in dairy cattle is a metabolic disorder. Primary or secondary ketosis can result during early lactation (McSherry et al., 1960; Grummer, 1993). Generally, high milk production during lactation or inadequate energy intakes result in a negative energy balance (**NEB**), which induces ketosis (Grummer, 1995). The clinical features of ketotic cows include anorexia, depression, and metabolic disorders (Grummer, 1993; Grummer, 1995; Baird, 1982; Herdt, 2000). Additionally, ketotic cows have low milk production and poor reproductive capacity (Baird, 1982). Because ketosis can lead to substantial economic losses in the dairy industry, it is of utmost importance to prevent ketosis in dairy cattle.

Metabolomics is the scientific analysis of the chemical processes that lead to the production of metabolites. Metabolomics provides a platform for the investigation of drug toxicity, disease processes, and altered gene function. Metabolomics can be performed in biological samples for a thorough and quantitative analysis of metabolites (Nicholson et al., 1999). Common metabolomic technologies include nuclear magnetic resonance (**NMR**), GC-MS, LC-MS, and metabolites chip (Nicholson et al., 2002). Of these techniques, ¹H-NMR has important advantages, including its ability to provide unbiased and rapid results on a wide range of small metabolites. In addition, ¹H NMR does not require chromatographic separation, making it a faster method than GC-MS and LC-MS (Tikunov et al., 2010).

Several metabolomic studies have been reported in humans and rats with metabolic diseases (Mantle et al., 2011; Ouyang et al., 2011; Verwaest et al., 2011; Qi et al., 2012). Studies on the metabolomics of ketosis have been reported using GC-MS and NMR (Klein et al., 2012). However, little is known about plasma metabolomics in clinical and subclinical ketosis. The objective of the current study was to use ¹H NMR metabolomics to understand clinical and subclinical ketosis in dairy cattle. Our study will provide valuable information on the pathogenesis and biomarkers of ketosis.

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¹Corresponding author: zhy478@163.com

MATERIALS AND METHODS

Sample Collection

All experimental animals were treated according to the International Guiding Principles for Biomedical Research Involving Animals (Demers et al., 2006). In the current study, 24 cows with clinical ketosis (**CK**), 32 cows with subclinical ketosis (**SK**), and 24 healthy control cows (**C**) were selected from a dairy cattle farm in Mishan, Heilongjiang, China. Cows with typical clinical signs of ketosis and plasma BHBA concentrations >1.60 mmol/L were considered to have CK. Cows with few clinical signs and plasma BHBA concentrations >1.20 mmol/L were considered to have SK. Cows with no clinical signs and normal plasma BHBA concentrations (i.e., <1.00 mmol/L) were considered to be healthy controls (Al-Rawashdeh, 1999; Oetzel, 2004). All cows were fed a TMR during the transition period. The TMR consisted of 8.5 kg of concentrated feed, 18.5 kg of silage maize, 4 kg of hay, and 0.35 kg of fat. The TMR consisted of 55.60% DM, 16% CP, 1.75 mcal/DM of net energy for lactation, 5.60% fat, 39.10% NFD, 20.30% ADF, 180 g of Ca, and 116 g of P.

After an overnight fast, blood samples were collected from the vena caudalis mediana. Blood samples were stabilized in sodium heparin and immediately centrifuged at $1,400 \times g$ for 10 min at room temperature. The resulting supernatants were transferred to Eppendorf tubes (1 mL of plasma/tube) and stored at -80°C . Both clinical parameters and plasma metabolomics were measured in all experimental animals. The clinical parameters, which included age, parity, milk yield, blood glucose, BHBA, triglycerides, aspartate aminotransferase, and cholesterol, are shown in Table 1.

Metabolic Profile of Plasma by ^1H NMR

Plasma samples were allowed to thaw at room temperature. Approximately 400 μL of plasma was mixed

with 170 μL of deuterium oxide. The solution was centrifuged at $12,000 \times g$ for 10 min at 4°C . Aliquots (500 μL) of the resulting supernatants were transferred to 5-mm NMR tubes. All NMR spectra were recorded at 25°C in a Varian Unity INOVA 600 NMR spectrometer (Varian Inc., Palo Alto, CA). One-dimensional spectra were recorded using the Carr-Purcell-Meiboom-Gill sequence (Nicholson et al., 1995; Viant, 2003) with a spin-spin relaxation delay of 2.1 s and a spectral width of 8,000 Hz.

Data Processing and Statistical Analyses

All free induction decays were zero-filled to 64-K points and multiplied by an exponential function to a 1.0 Hz line-broadening factor before Fourier transformation. The ^1H NMR spectra were manually phased and baseline-corrected using TopSpin software (V3.0 Bruker Biospin, Karlsruhe, Germany; Ouyang et al., 2011). The ^1H NMR spectra of plasma samples were referenced to an internal lactic acid CH_3 resonance at δ 1.33. The ^1H NMR spectra were segmented into consecutive nonoverlapping regions of δ 0.002 chemical shift bins between δ 0.5 and 9.0. The spectral area within each bin was integrated. The bin size of δ 0.002 was chosen as the primary chemical shift bin width to avoid the encompassment of several peaks and to have clear peak multiplicities, which facilitate the identification of metabolites (Purohit et al., 2004). The water resonance (δ 4.35–5.39) and urea resonance (δ 5.40–6.50) regions were excluded to eliminate baseline effects. The peak area of each bin was calculated. Data were normalized using TopSpin by dividing each integrated segment by the total area of the spectrum to compensate for the concentration differences among the plasma samples.

For multivariate statistical analyses, the normalized data were analyzed using SIMCA-P software (Version 11.0, Umetrics AB, Umea, Sweden). Multivariate statistical analyses included principal component analysis (**PCA**), partial least squares discriminant

Table 1. Clinical data and biochemical parameters of dairy cows (mean $\pm$ SD)

Parameter	Clinical ketosis	Subclinical ketosis	Control
Number	24	33	24
Postpartum days	12 $\pm$ 5	14 $\pm$ 6	16 $\pm$ 6
Age (yr)	3 $\pm$ 1	3 $\pm$ 1	4 $\pm$ 2
Parity	2 $\pm$ 1	2 $\pm$ 1	2 $\pm$ 1
Milk yield (kg/d)	23.82 $\pm$ 7.55	23.07 $\pm$ 7.38	23.00 $\pm$ 1.68
Glucose (mmol/L)	1.90 $\pm$ 0.70 ^a	2.70 $\pm$ 0.63 ^{ab}	3.37 $\pm$ 0.58
BHBA (mmol/L)	2.49 $\pm$ 0.60 ^a	1.22 $\pm$ 0.17 ^{ab}	0.82 $\pm$ 0.12
Cholesterol (mmol/L)	2.36 $\pm$ 0.70	2.45 $\pm$ 0.68	1.98 $\pm$ 0.80
Aminotransferase (U/L)	113.13 $\pm$ 23.74	113.29 $\pm$ 27.46	102.58 $\pm$ 28.58
Triglycerides (mmol/L)	0.13 $\pm$ 0.40	0.16 $\pm$ 0.50	0.13 $\pm$ 0.57

^{a,b}Different superscripts within a column represent significant differences ($P < 0.05$).

analysis (**PLS-DA**), and orthogonal partial least-squares discriminant analysis (**OPLS-DA**). Analysis of the metabolite signals in the ^1H NMR spectra was first performed with PCA, which displays the internal structure of data sets in an unbiased manner and reduces data dimensionality (Wold et al., 1987; Trygg and Wold, 2002; Trygg et al., 2007). A PCA scores plot was used to reveal the presence of outliers outside the 95% significance region of Hotelling's T^2 ellipse (i.e., strong outliers).

Data were analyzed by PCA, PLS-DA, and OPLS-DA following the removal of outliers by PCA pretreatment to assess variances among predefined groups and to remove irrelevant factors for the identification of metabolites (Trygg and Wold, 2002). The PLS-DA was based on a unit variance scaling strategy. A 10-fold cross-validation, which was used to validate the PLS-DA model according to the default software settings, was performed to evaluate the quality of the model by 3 parameters (i.e., R^2X , R^2Y , and Q^2). The R^2X parameters, which describe the optimization of the analytical model, represent the total variation in X. The R^2Y parameters describe the variation in the response variable Y. The Q^2 parameter, which represents the predictive ability of the model, represents the authenticity of the predicted results. The corresponding scores plots and verification plots were used to identify differences in metabolites among the 3 groups. On the scores plot, each point represented an individual sample. The center of each ellipse and the margin indicate mean and standard deviation, respectively. A permutation test from the verification plots ($n = 200$) was performed to validate and test the degree of over-fitting for the PLS-DA model. The correlation coefficient between the original Y and the permuted Y was plotted against the cumulative R^2 and Q^2 ; a regression line was calculated with the R^2 - and Q^2 -intercept limits. These tests compared the goodness-of-fit of the original model with the goodness-of-fit of several models based on data in which the order of the Y observations was randomly permuted while the X matrix remained constant (Ni et al., 2008). In the verification plots, the y-axis represents R^2Y (green triangle) and Q^2Y (blue box) and the x-axis designates the Pearson correlation coefficient between the original and permuted rank-ripeness. When the Q^2 regression line had a negative intercept and R^2 values on the left were lower than the original points on the right, the validation plots of the PLS-DA model were effective.

As an extension of the PLS-DA model and featuring integrated orthogonal signal correction, OPLS-DA models were generated using the first principal component (**t[1]P**) and the second orthogonal component (Yin et al., 2008). The OPLS-DA was used

to maximize the covariance between the measured data (X variable, peak intensities in NMR spectra) and the response variable (Y variable, predictive classifications) and to simultaneously remove uncorrelated variations between the X and Y variables or X variables that were orthogonal to Y variables (Sui et al., 2012). Scores plots combining the reliability and correlation from the OPLS-DA models were used to identify differential metabolites among the groups. The relevant metabolites for each separation model and the metabolite differences among the groups were shown as coefficient of variation plots. The significance of the scores plots obtained in OPLS-DA are similar to those obtained in PLS-DA. The OPLS-DA is also based on unit variance scaling strategy. A 10-fold cross-validation was used to obtain Q^2 and R^2 values, which represents the predictive ability of the model and the explained variance, respectively. Sensitivity, specificity, and classification rates (i.e., percentage of samples correctly classified), which were obtained within a 10-fold cross-validation of OPLS-DA models among groups, were calculated.

The relevant metabolites for each separation model and the metabolite differences among the groups are shown as color-coded coefficient plots of OPLS-DA. By assessing the coefficient plots, the differences in metabolites among the groups can be determined. In the color-coded coefficient plots, signals with a positive direction of **t[1]P** corresponded to metabolites that were present at high concentrations. The negative direction of **t[1]P** indicated a negative direction of peak values among the groups. Moreover, signals in the spectra corresponded to metabolites in a given group. The color in each coefficient plot represented the strength of the metabolites in explaining the scores. For instance, red indicates a more significant contribution to the separation between the groups than blue.

The correlation coefficient [$\text{Corr}(t, X)$] of ± 0.396 was adopted as a cut-off value to select the variables that were most significantly correlated with the OPLS-DA discriminant scores (Sui et al., 2012). The correlation coefficients were calculated by Pearson linear correlation coefficients using Matlab R2007a software (MathWorks Inc., Natick, MA). The higher the $|r|$ values, the bigger the difference. Conversely, the lower the $|r|$ values, the smaller the difference. Different metabolites among the 3 groups were identified by correlation coefficients. Finally, to select potential biomarkers, the different metabolites were validated using Student's *t*-test. Age, parity, and MY data were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL). Values are expressed as mean $\pm$ SD. Multiple comparisons were performed by one-way ANOVA. Statistical significance was set at $P < 0.05$.

RESULTS

Clinical Information

The clinical data from the CK, SK, and C groups revealed biochemical differences among the groups (Table 1). The differences among the 3 groups in age, parity, milk yield, and cholesterol, aminotransferase, and triglycerides plasma levels were not significant ($P > 0.05$). However, BHBA and glucose plasma levels were significantly different among the 3 groups ($P < 0.05$).

^1H NMR Spectroscopy of Plasma Samples

Representative ^1H NMR spectra (δ 0.5–5.5 and 6.5–9.0) of plasma samples from the CK, SK, and C groups are shown in Figure 1. Data obtained from the ^1H NMR spectra were analyzed; the metabolites were identified based on data obtained from previous studies (Nicholson et al., 1995). The data were subsequently analyzed using multivariate statistics (PCA, PLS-DA, and OPLS-DA).

PCA to Detect Outliers

The resulting scores plot (Figure 2a; $R^2X = 0.768$, $Q^2 = 0.610$) of PCA was obtained with PC1 and PC2, which represented 54.3 and 22.4% variance, respectively. The scores plot of PCA was shown in Figure 2a. Five

outliers from the CK group and 1 outlier from the C group were spotted outside the 95% significance region of Hotelling's T^2 ellipse. The strongest 3 signals in the original spectra of the CK and C groups, respectively, were corresponded to acetone, BHBA, and acetate. Conversely, lactate and glucose were identified as the 2 weakest signals in the original spectra. The changes of 5 outliers in the CK group were not associated with their biological nature, so they were not excluded in subsequent analyses. However, the changes of original spectra in C group were abnormal; one outlier from the C group was excluded in subsequent analyses.

After removing the outlier from the C group, PCA was performed (Figure 2b; $R^2X = 0.771$, $Q^2 = 0.658$) with the first 2 PC (PC1 and PC2), which represented 60.4 and 16.6% variance, respectively. Significant differences ($P < 0.05$) were observed in the CK and SK groups on PC1 scores; however, no significant differences ($P > 0.05$) were observed on PC2 scores (Figure 2b).

Discrimination Between Normal and Pathological Samples Using PLS-DA

A PLS-DA was used to analyze the ^1H NMR spectra. The scores plots and validation plots of PLS-DA are shown in Figure 3. Based on the PLS-DA models (scores plots in Figure 3a–c), the CK and C groups

Figure 1. Representative 600 MHz ^1H -nuclear magnetic resonance spectra (δ 0.5–5.5 and δ 6.5–9.0) of plasma samples obtained from (A) clinical ketosis group, (B) subclinical ketosis group, or (C) control group. The δ 6.5 to 9.0 region (dashed box) was magnified 32 times relative to the δ 0.5 to 5.5 region for clarity purposes. The total spin-echo time was 100 ms. 1-MH = 1-methylhistidine; 3-HB = BHBA; 3-MH = 3-methylhistidine; AcAc = acetoacetate; Ace = acetate; Act = acetone; All = allanotin; Cho = choline; Cit = citrate; Cr = creatine; DMA = dimethylamine; For = formate; GPC = glycerolphosphocholine; L1 = low-density lipoprotein and very low-density lipoprotein, $\text{CH}_3-(\text{CH}_2)_n$; L2 = low-density lipoprotein and very low-density lipoprotein, $\text{CH}_3-(\text{CH}_2)_n$; L3 = lipid, $=\text{CH}-\text{CH}_2-\text{CH}=\text{}$; Lac = lactate; *m*-I = *myo*-Inositol; NAG = N-acetyl glycoprotein; PC = phosphocholine; U = unassigned resonance; α -Glc = α -glucose; β -Glc = β -glucose.

Figure 2. (a) Principal component analysis (PCA) scores plot based on ^1H -nuclear magnetic resonance spectra of plasma samples obtained from the 3 groups. On the scores plot, each point represents an individual sample. Group 1 = clinical ketosis (CK; ■); group 2 = subclinical ketosis (SK; ●); group 3 = control (C; ◆). The center and margin of the ellipse indicate mean and standard deviation, respectively. (b) The PCA scores plot of plasma samples obtained from the 3 groups without outliers. On the scores plot, each point represents an individual sample. Group 1 = CK (■); group 2 = SK (●); group 3 = C (◆). The center and margin of the ellipse indicate mean and standard deviation, respectively. Color version available in the online PDF.

were discriminated with an R^2X of 0.307, an R^2Y of 0.853, and a Q^2 of 0.698. The SK and C groups had an R^2X of 0.250, an R^2Y of 0.739, and a Q^2 of 0.529. Finally, the CK and SK groups had an R^2X of 0.246, an R^2Y of 0.779, and a Q^2 of 0.490. The goodness-of-fit (R^2 and Q^2) of the original PLS-DA models and cluster of 200 Y-permuted models were shown in the validation plots of Figure 3a–c. The results showed that the original PLS-DA models were effective.

Discrimination Between Normal and Pathological Samples Using OPLS-DA

Based on the OPLS-DA models, the CK and C groups were discriminated with $R^2X = 0.307$ and $Q^2 = 0.706$; the SK and C groups were discriminated with $R^2X = 0.251$ and $Q^2 = 0.510$; and the CK and SK groups were discriminated with $R^2X = 0.246$ and $Q^2 = 0.462$. In OPLS-DA scores plots, significant biochemical differences were observed between the CK and C groups (Figure 4a) and between the SK and C groups (Figure 4b). In addition, some degree of separation between the CK and SK groups could be observed (Figure 4c). By assessing the coefficient plots of OPLS-DA (Figure 4), the main metabolites contributing to the separation of the 3 groups were shown in Table 2. Among the 25 metabolites, 4 were upregulated, 7 were downregulated, and 14 were both upregulated and downregulated. The expression levels of plasma His, glutamate, Gln, Lys, Phe, blood glucose, and lactate were significantly downregulated in cows with ketosis. The expression levels of *myo*-inositol, formate, citrate, Ala, Pro, Tyr, low-density lipoprotein (LDL), and very low density lipoprotein (VLDL) were downregulated only in cows with clinical ketosis. However, the expression levels of acetate, NAG, BHBA, acetoacetate, and acetone were significantly upregulated in cows with ketosis. Choline, creatine, Gly, Leu, Ile, and Val were upregulated only in cows with subclinical ketosis. The most relevant metabolic differences between the CK, SK, and C groups are described in Figure 5.

Table 3 shows the sensitivity, specificity, and classification rates of the models among groups. The OPLS-DA models were able to predict clinical ketosis with a sensitivity of 100% and a specificity of 100%. The OPLS-DA models were able to predict subclinical ketosis with a sensitivity of 97.0% and a specificity of 95.7%. The results revealed that the OPLS-DA models can identify differences in metabolites among the CK, SK, and C groups.

DISCUSSION

Studies have shown that clinical ketosis in dairy cows generally occurs between wk 2 and 7 after calving (Baird, 1982; Grummer, 1993, 1995; Herdt, 2000). However, most cows suffer from subclinical ketosis during early lactation. Subclinical ketosis is characterized by an increased production of ketone bodies without clinical signs of the disease (Duffield et al., 1997). ^1H -Nuclear magnetic resonance spectroscopy is a powerful tool for the identification of metabolites from multiple biological samples. In the current study, NMR-based metabolomics were used to identify plasma metabolites

Figure 3. (a) Scores plot (top) and validation plot (bottom) of partial least squares discriminant analysis (PLS-DA) models from the clinical ketosis (CK) and control (C) groups. Group 1 = CK (■); group 3 = C (●); R² (▲); Q² (■). (b) Scores plot (top) and validation plot (bottom) of PLS-DA models from the subclinical ketosis (SK) and C groups. Group 2 = SK (■); group 3 = C (●); R² (▲); Q² (■). (c) Scores plot (top) and validation plot (bottom) of PLS-DA models from the CK and SK groups. Group 1 = CK (■); group 2 = SK (●); R² (▲); Q² (■). Color version available in the online PDF.

from dairy cows with ketosis. The experimental results clearly showed that significant differences exist in plasma ¹H NMR spectra of cows with and without ketosis. Combined with statistical models, ¹H NMR allowed the identification of characteristic metabolites of ketosis.

Three metabolites (acetoacetate, BHBA, and acetone) increased in both the CK and SK groups. However, plasma glucose levels in the CK and SK groups were lower than in the C group. These results (Table 2) were consistent with the experimental results shown in Table 1. Additionally, these results confirmed that the clinical characteristics of ketosis include high concentrations of ketone bodies and low plasma glucose levels (Duffield et al., 1997; González et al., 2011).

As a result of milk production and limited feed intake, high-yielding dairy cows usually experience NEB during early lactation (Nielen et al., 1994). Negative energy balance leads to increased gluconeogenesis and mobilization of body triglycerides and protein. Different from single-stomach animals, glucose regulation is very complicated in ruminant animals. Because of the

lack of carbohydrates in the rumen or increasing energy requirements, ruminants mainly rely on noncarbohydrate compounds to meet their energy demands via gluconeogenesis (Nafikov and Beitz, 2007).

In ruminants, the principal gluconeogenic substrates include propionic acid, lactic acid, glycerol, and gluconeogenic amino acids (e.g., glutamate, aspartic acid, Gly, His, Pro, Gln, and Val; Gerich et al., 2001). In the liver, lactate is converted into pyruvic acid by the enzyme lactate dehydrogenase. As the first gluconeogenic substrate, pyruvic acid is further converted to glucose via gluconeogenesis. Other gluconeogenic amino acids and intermediate products of the tricarboxylic acid (TCA) cycle can be used as gluconeogenic substrates by producing oxaloacetate. Some amino acids, such as Phe and Tyr, are both gluconeogenic and ketogenic. In the current experiment, lactate, glutamate, Gln, and Phe levels in CK and SK were higher than in C, suggesting that a lack of gluconeogenic substrates is an important risk factor in the pathogenesis of ketosis. However, Ala, Pro, Gly, Val, and Tyr levels were lower

Figure 4. (a) Orthogonal partial least-squares discriminant analysis (OPLS-DA) scores plot (top) derived from ^1H -nuclear magnetic resonance (NMR) spectra of plasma samples and the corresponding coefficient plots (center and bottom plots) obtained from the clinical ketosis (CK) and control (C) groups. Group 1 = CK (■); group 3 = C (●). The color map shows significant differences in metabolites between the 2 groups. (b) The OPLS-DA scores plot (top) derived from ^1H -NMR spectra of plasma samples and the corresponding coefficient plots (center and bottom plots) obtained from the subclinical ketosis (SK) and C groups. Group 2 = SK (■); group 3 = C (●). The color map shows significant differences in metabolites between the 2 groups. (c) The OPLS-DA scores plot (top) derived from ^1H -NMR spectra of plasma samples and the corresponding coefficient plots (center and bottom plots) obtained from the CK and SK groups. Group 1 = CK (■); group 2 = SK (●). The color map shows the significant differences in metabolites between the 2 groups. $t[2]O$ = second orthogonal component; $t[1]P$ = first principal component. Color version available in the online PDF.

in the CK group than in the other 2 groups. This result suggests that these amino acids play important roles in clinical ketosis. When dairy cows have severe NEB during early lactation, the mobilization of triglycerides from adipose tissue increases to meet the energy demands. Additionally, a large amount of NEFA is also produced and incorporated in the liver (Xu et al., 2008).

In the liver, NEFA can be converted into acetyl CoA to supply energy via the TCA cycle. If propionic acid is insufficient for the TCA cycle as a gluconeogenic substrate, excess NEFA in the liver can be converted into ketone bodies, thereby contributing to ketosis (Drack-

ley et al., 1992). Ketogenic amino acids (e.g., Leu, Ile, and Lys) can also enter the TCA cycle by oxidative deamination. Once deaminated, the amino acids are converted into acetoacetic acid with the participation of acetyl CoA (Yudkoff et al., 2005; Grassi et al., 2012). In the current experiment, Leu and Ile levels in the SK group were higher than in the CK and C groups; acetate and Lys levels in the CK and SK groups were lower than in the C group. However, citric acid levels were only low in the CK group, suggesting that ketotic cows consume large amounts of ketogenic substances, especially amino acids, for energy. A disturbance in the TCA cycle may contribute to clinical ketosis

Table 2. Orthogonal partial least-squares discriminant analysis coefficients derived from ^1H -nuclear magnetic resonance (NMR) data of plasma metabolites obtained from the clinical ketosis (CK), subclinical ketosis (SK), and control (C) groups

No.	Metabolite	^1H chemical shifts and multiplicity ¹ (δ)	CK ² ($\times 10^{-4}$)	SK ² ($\times 10^{-4}$)	C ² ($\times 10^{-4}$)	r^3		
						CK-C	SK-C	CK-SK
1	1-Methylhistidine	7.06(s), 7.78(s)	0.4 ± 0.1^a	0.6 ± 0.1^{ab}	0.6 ± 0.1	0.638	0.509	0.544
2	BHBA	1.20(d), 2.31(dd), 2.41(dd), 4.16(m)	14.6 ± 4.9^a	80.0 ± 2.0^{ab}	4.7 ± 1.8	-0.945	-0.907	-0.781
3	3-Methylhistidine	6.99(s), 7.74(s)	0.4 ± 0.1^a	0.6 ± 0.1^b	0.7 ± 0.3	0.611	0.463	0.723
4	Acetate	1.92(s)	25.1 ± 16.9^a	16.8 ± 5.0	13.2 ± 8.4	-0.499	-0.487	—
5	Acetoacetate	2.28(s)	8.3 ± 3.7^a	4.9 ± 1.3^{ab}	3.8 ± 0.8	-0.862	-0.823	-0.651
6	Acetone	2.23(s)	21.1 ± 15.1^a	7.0 ± 4.4^{ab}	2.3 ± 3.0	-0.881	-0.803	-0.718
7	Alanine	1.48(d)	4.1 ± 1.3^a	5.3 ± 1.0^b	5.7 ± 1.4	0.642	—	0.522
8	Allantoin	5.39(s)	1.1 ± 0.6	1.2 ± 0.4	1.0 ± 0.4	—	-0.430	—
9	Choline	3.20(s)	5.0 ± 0.7	5.4 ± 0.6^b	5.0 ± 1.0	—	-0.444	0.418
10	Citrate	2.54(d), 2.68(d)	0.9 ± 0.2	1.1 ± 0.3^b	1.1 ± 0.5	0.589	—	0.560
11	Creatine	3.04(s), 3.93(s)	7.3 ± 1.2	8.2 ± 1.0^{ab}	7.5 ± 1.4	—	-0.542	0.585
12	Formate	8.46(s)	0.2 ± 0.1^a	0.3 ± 0.1^b	0.3 ± 0.1	0.645	—	0.516
13	Glutamate	2.10(m), 2.35(m), 3.78(t)	3.0 ± 0.3^a	3.4 ± 0.3^{ab}	3.7 ± 0.4	0.839	0.683	0.657
14	Glutamine	2.14(m), 2.45(m), 3.78(t)	2.4 ± 0.5^a	2.9 ± 0.7^b	2.9 ± 0.7	0.839	0.683	0.657
15	Glycine	3.56(s)	15.0 ± 3.8^a	16.6 ± 3.1^a	13.0 ± 3.1	—	-0.589	0.449
16	Isoleucine	0.94(t), 1.01(d)	8.7 ± 1.1	9.7 ± 1.0^{ab}	9.0 ± 0.9	0.590	-0.647	0.777
17	CH ₃ -(CH ₂) _n -(low-density lipoprotein and very low-density lipoprotein)	0.86(br)	12.4 ± 1.9	13.4 ± 2.5	12.8 ± 3.7	0.473	—	0.401
18	Lactate	1.33(d), 4.12(q)	8.9 ± 3.5^a	10.3 ± 4.4^a	16.0 ± 8.0	0.740	0.571	0.437
19	Leucine	0.96(d)	14.4 ± 2.4	15.8 ± 2.3^{ab}	13.9 ± 2.2	—	-0.540	0.470
20	Lysine	1.71(m), 1.90(m), 3.01(m), 3.76(t)	2.7 ± 0.3^a	3.3 ± 0.2^{ab}	3.5 ± 0.5	0.793	0.573	0.821
21	<i>myo</i> -Inositol	3.59(dd), 3.66(dd), 4.06(m)	5.8 ± 0.9	6.5 ± 0.6^{ab}	6.0 ± 0.9	0.667	-0.419	0.596
22	N-acetyl glycoprotein signals	2.04(s)	10.3 ± 1.3^a	10.8 ± 1.6^a	9.0 ± 2.1	-0.581	-0.591	0.659
23	Phenylalanine	7.32(m), 7.37(m), 7.42(m)	0.6 ± 0.1^a	0.7 ± 0.1^b	0.8 ± 0.3	0.668	0.426	0.696
24	Proline	2.01(m), 2.08(m)	4.5 ± 0.5^a	5.1 ± 0.4^b	5.1 ± 0.4	0.699	—	0.651
25	Tyrosine	6.90(d), 7.19(d)	0.3 ± 0.1^a	0.4 ± 0.1^b	0.5 ± 0.1	0.752	—	0.729
26	U1	1.17(d)	4.1 ± 2.9^a	2.6 ± 0.6	2.6 ± 0.6	-0.582	-0.592	-0.519
27	U2	3.15(s)	1.9 ± 0.8	2.6 ± 0.8^{ab}	1.9 ± 1.1	—	—	0.530
28	Valine	0.99(d), 1.04(d)	9.1 ± 1.7	10.1 ± 1.6^{ab}	8.8 ± 1.6	—	-0.625	0.513
29	α -Glucose	3.42(t), 3.54(dd), 3.71(t), 3.73(m), 3.84(m), 5.23(d)	12.6 ± 3.3^a	15.0 ± 1.8^{ab}	16.9 ± 2.9	0.791	0.689	0.647
30	β -Glucose	3.25(dd), 3.41(t), 3.46(m), 3.49(t), 3.90(dd), 4.65(d)	14.9 ± 3.6^a	17.9 ± 2.1^{ab}	20.2 ± 2.9	0.818	0.715	0.644

^{a,b}Different superscripts within a column represent significant difference ($P < 0.05$).

¹Chemical shifts (δ) denote the position of a signal in the NMR spectra; the multiplicity describes the coupling pattern of an NMR signal. s = singlet; d = doublet; t = triplet; q = quartet; dd = doublet of doublets; m = multiplet; br = broad resonance. Data were used for metabolite identification.

²Data of peak intensity are expressed in mean $\pm$ SD.

³Correlation coefficients: positive and negative signs indicate positive and negative correlation in concentrations, respectively. The correlation coefficient $|r| > 0.396$ was used as the cut-off value for statistical significance based on the discrimination significance at the level of $P = 0.05$ and degree of freedom = 23. An em dash means the correlation coefficient $|r| < 0.396$.

Table 3. Sensitivity, specificity, and classification rates calculated from cross-validated orthogonal partial least-squares discriminant analysis of plasma samples of 2 arbitrary groups

Item	Clinical ketosis-control	Subclinical ketosis-contol	Clinical ketosis- Subclinical ketosis
Sensitivity (%)	100	97.0	95.8
Specificity (%)	100	95.7	97.0
Classification rate (%)	100	96.4	96.5

In the liver, NEFA can be completely oxidized for energy; NEFA can also be used to form triglycerides by esterification. Triglycerides can then be incorporated into VLDL, which are transported to mammary tissue for lactation or converted into LDL in the bloodstream (Grummer, 2008; Xu et al., 2008). In the liver, choline, a component of lecithin, can help transport fatty acids and promote fatty acid oxidation to prevent excessive

fat accumulation in hepatocytes (Gao et al., 2008). Compared with the C group, the CK group had lower plasma VLDL and LDL levels. However, choline was higher in the SK group than in the C group, which suggests that ketosis may negatively affect triglycerides mobilization outside the liver, thereby contributing to fatty liver.

Inositol is a carbohydrate; it exists in 9 stereoisomers, the most predominant form being *myo*-inositol. Inositol is an important component of structural lipids, such as phosphatidylinositol, and its various phosphate lipids (e.g., phosphatidylinositol phosphate). *Myo*-inositol forms the structural basis of several inositol phosphates. Inositol and some of its mono- and polyphosphates are secondary messenger molecules involved in cell signaling. Additionally, they are involved in fat lipolysis and have blood cholesterol-lowering effects (Rapiejko et al., 1986). In the current study, *myo*-inositol was only present in the CK group, suggesting that an increased fat

Figure 5. This schematic representation depicts the most relevant metabolic differences among the clinical ketosis (CK), subclinical ketosis (SK), and control groups (C). Parenthetical notation (C, S) represents the changes only found in clinical ketosis and subclinical ketosis, respectively. LDL = low-density lipoprotein; VLDL = very low density lipoprotein. Color version available in the online PDF.

mobilization may require more *myo*-inositol to maintain fat metabolism; however, a decrease in *myo*-inositol may interfere with fat metabolism in clinical ketosis.

Low plasma histidine levels may be closely related to protein waste, chronic inflammation, and oxidative stress (Kumar et al., 2010). In the current experiment, 1-methyl histidine and 3-methyl histidine decreased in the CK group compared with the SK and C groups. This result implies that low histidine levels in ketotic cows can lead to the development of infectious diseases.

During nucleic acid biosynthesis, formate can provide a carbon source to N5, N10-methylenetetrahydrofolate (Litsanov et al., 2012). In the current study, formate was lower in the CK group than in the SK and C groups, suggesting that cows with clinical ketosis would be more vulnerable to inflammatory diseases; formate in large amounts can be used to treat inflammatory conditions (Duffield et al., 1998) involved in purine nucleotide biosynthesis (Lin et al., 2009), such as mastitis.

Creatine plays an important role in energy balance when animals have high energy requirements. Blood creatine may be incorporated into cells by a creatine transporter (Ireland et al., 2009). In the current study, creatine levels in the CK group were higher than in the SK group. This result implies that creatine as an energy buffer may be involved in the regulation of energy metabolism in clinical ketosis.

In summary, the systemic changes in endogenous metabolites from ketosis mainly affected energy, amino acid, lipid, and carbohydrate metabolism. Metabolite profiling, obtained from ^1H NMR-based metabolomics analyses of serum samples from ketotic cows, clearly distinguished clinical and subclinical ketosis from healthy controls. Therefore, ^1H NMR-based metabolomics is a novel method for detecting ketosis before symptoms develop. Metabolomics may assist in early diagnosis, adequate therapies, and better clinical management of the disease.

CONCLUSIONS

^1H -Nuclear magnetic resonance-based plasma metabolomics of cows with clinical ketosis and subclinical ketosis can be used to understand the metabolic changes that occur in the pathogenesis of the disease. Furthermore, the different metabolites in the CK, SK and C groups contribute to a better insight of the pathogenesis of ketosis. Future studies should focus on other metabolomic technologies and biomarker validations to further understand ketosis.

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REFERENCES

- Al-Rawashdeh, O. F. 1999. Prevalence of ketonemia and associations with herd size, lactation stage, parity and postparturient diseases in Jordanian dairy cattle. *Prev. Vet. Med.* 40:117–125.
- Baird, G. D. 1982. Primary ketosis in the high-producing dairy cow: Clinical and subclinical disorders, treatment, prevention, and outlook. *J. Dairy Sci.* 65:1–10.
- Demers, G., G. Griffin, G. De Vroey, J. R. Haywood, J. Zurlo, and M. Bédard. 2006. Harmonization of animal care and use guidance. *Science* 312:700–701.
- Drackley, J. K., M. J. Richard, D. C. Beitz, and J. W. Young. 1992. Metabolic changes in dairy cows with ketonemia in response to feed restriction and dietary 1,3-butanediol. *J. Dairy Sci.* 75:1622–1634.
- Duffield, T. F., D. F. Kelton, K. E. Leslie, K. D. Lissimore, and J. H. Lumsden. 1997. Use of test day milk fat and milk protein to detect subclinical ketosis in dairy cattle in Ontario. *Can. Vet. J.* 38:713–718.
- Duffield, T. F., D. Sandals, K. E. Leslie, K. Lissimore, B. W. McBride, J. H. Lumsden, P. Dick, and R. Bagg. 1998. Efficacy of monensin for the prevention of subclinical ketosis in lactating dairy cows. *J. Dairy Sci.* 81:2866–2873.
- Gao, H., B. Dong, X. Liu, H. Xuan, Y. Huang, and D. Lin. 2008. Metabonomic profiling of renal cell carcinoma: High-resolution proton nuclear magnetic resonance spectroscopy of human serum with multivariate data analysis. *Anal. Chim. Acta* 624:269–277.
- Gerich, J. E., C. Meyer, H. J. Woerle, and M. Stumvoll. 2001. Renal gluconeogenesis: Its importance in human glucose homeostasis. *Diabetes Care* 24:382–391.
- González, F. D., R. Muñio, V. Pereira, R. Campos, and J. L. Bedito. 2011. Relationship among blood indicators of lipomobilization and hepatic function during early lactation in high-yielding dairy cows. *J. Vet. Sci.* 12:251–255.
- Grassi, I., C. Nanni, V. Allegri, J. J. Morigi, G. C. Montini, P. Castellucci, and S. Fanti. 2012. The clinical use of PET with (11) C-acetate. *Am. J. Nucl. Med. Mol. Imaging* 2:33–47.
- Grummer, R. R. 1993. Etiology of lipid-related metabolic disorders in periparturient dairy cows. *J. Dairy Sci.* 76:3882–3896.
- Grummer, R. R. 1995. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. *J. Anim. Sci.* 73:2820–2833.
- Grummer, R. R. 2008. Nutritional and management strategies for the prevention of fatty liver in dairy cattle. *Vet. J.* 176:10–20.
- Herd, T. H. 2000. Ruminant adaptation to negative energy balance. Influences on the etiology of ketosis and fatty liver. *Vet. Clin. North Am. Food Anim. Pract.* 16:215–230.
- Ireland, Z., A. P. Russell, T. Wallimann, D. W. Walker, and R. Snow. 2009. Developmental changes in the expression of creatine synthesizing enzymes and creatine transporter in a precocial rodent, the spiny mouse. *BMC Dev. Biol.* 9:39.
- Klein, M. S., N. Buttcher, S. P. Miemczyk, A. K. Immervoll, C. Louis, S. Wiedemann, W. Junge, G. Thaller, P. J. Oefner, and W. Gronwald. 2012. NMR metabolomic analysis of dairy cows reveals milk glycerophosphocholine to phosphocholine ratio as prognostic biomarker for risk of ketosis. *J. Proteome Res.* 11:1373–1381.

- Kumar, A., L. Bala, J. Kalita, U. K. Misra, R. L. Singh, C. L. Khetrapal, and G. N. Babu. 2010. Metabolomic analysis of serum by (1) H NMR spectroscopy in amyotrophic lateral sclerosis. *Clin. Chim. Acta* 411:563–567.
- Lin, Z. Y., P. B. Xu, S. K. Yan, H. B. Meng, G. J. Yang, W. X. Dai, X. R. Liu, J. B. Li, X. M. Deng, and W. D. Zhang. 2009. A metabolomic approach to early prognostic evaluation of experimental sepsis by (1)H NMR and pattern recognition. *NMR Biomed.* 22:601–608.
- Litsanov, B., M. Brocker, and M. Bott. 2012. Toward homosuccinate fermentation: Metabolic engineering of corynebacterium glutamicum for anaerobic production of succinate from glucose and formate. *Appl. Environ. Microbiol.* 78:3325–3337.
- Mantle, P. G., A. W. Nicholls, and J. P. Shockcor. 2011. H NMR spectroscopy-based metabolomic assessment of uremic toxicity, with toxicological outcomes, in male rats following an acute, mid-life insult from ochratoxin a. *Toxins (Basel)* 3:504–519.
- McSherry, B. J., D. C. Maplesden, and H. D. Branion. 1960. Ketosis in cattle—A review. *Can. Vet. J.* 1:208–213.
- Nafikov, R. A., and D. C. Beitz. 2007. Carbohydrate and lipid metabolism in farm animals. *J. Nutr.* 137:702–705.
- Ni, Y., M. Su, J. Lin, X. Wang, Y. Qiu, A. Zhao, T. Chen, and W. Jia. 2008. Metabolic profiling reveals disorder of amino acid metabolism in four brain regions from a rat model of chronic unpredictable mild stress. *FEBS Lett.* 582:2627–2636.
- Nicholson, J. K., J. Connelly, J. C. Lindon, and E. Holmes. 2002. Metabonomics: A platform for studying drug toxicity and gene function. *Nat. Rev. Drug Discov.* 1:153–161.
- Nicholson, J. K., P. J. Foxall, M. Spraul, R. D. Farrant, and J. C. Lindon. 1995. 750 MHz ¹H and ¹H–¹³C NMR spectroscopy of human blood plasma. *Anal. Chem.* 67:793–811.
- Nicholson, J. K., J. C. Lindon, and E. Holmes. 1999. Metabonomics: Understanding the metabolic responses of living systems to pathological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 29:1181–1189.
- Nielen, M., M. G. Aarts, A. G. Jonkers, T. Wensing, and Y. H. Schukken. 1994. Evaluation of two cow-side tests for the detection of subclinical ketosis in dairy cows. *Can. Vet. J.* 35:229–232.
- Oetzel, G. R. 2004. Monitoring and testing dairy herds for metabolic disease. *Vet. Clin. North Am. Food Anim. Pract.* 20:651–674.
- Ouyang, X., Y. Dai, J. L. Wen, and L. X. Wang. 2011. ¹H NMR-based metabolomic study of metabolic profiling for systemic lupus erythematosus. *Lupus* 20:1411–1420.
- Purohit, P. V., D. M. Rocke, M. R. Viant, and D. L. Woodruff. 2004. Discrimination models using variance-stabilizing transformation of metabolomic NMR data. *OMICS* 8:118–130.
- Qi, S. W., Z. G. Tu, W. J. Peng, L. X. Wang, X. Ou-Yang, A. J. Cai, and Y. Dai. 2012. ¹H NMR-based serum metabolic profiling in compensated and decompensated cirrhosis. *World J. Gastroenterol.* 18:285–290.
- Rapiejko, P. J., J. K. Northup, T. Evans, J. E. Brown, and C. C. Malbon. 1986. G-proteins of fat-cells. Role in hormonal regulation of intracellular inositol 1,4,5-trisphosphate. *Biochem. J.* 240:35–40.
- Sui, W., L. P. Li, W. T. Che, G. M. Zuo, J. J. Chen, W. X. Li, and Y. Dai. 2012. A proton nuclear magnetic resonance-based metabolomics study of metabolic profiling in immunoglobulin A nephropathy. *Clinics* 67:363–373.
- Tikunov, A. P., C. B. Johnson, H. Lee, M. K. Stoskopf, and J. M. Macdonald. 2010. Metabolomic investigations of American oysters using ¹H-NMR spectroscopy. *Mar. Drugs* 8:2578–2596.
- Trygg, J., E. Holmes, and T. Lundstedt. 2007. Chemometrics in metabolomics. *J. Proteome Res.* 6:469–479.
- Trygg, J., and S. Wold. 2002. Orthogonal projections to latent structures (O-PLS). *J. Chemometr.* 16:119–128.
- Verwaest, K. A., T. N. Vu, K. Laukens, L. E. Clemens, H. P. Nguyen, B. Van Gasse, J. C. Martins, A. Van Der Linden, and R. Dommisse. 2011. ¹H NMR based metabolomics of CSF and blood serum: A metabolic profile for a transgenic rat model of Huntington disease. *Biochim. Biophys. Acta* 1812:1371–1379.
- Viant, M. R. 2003. Improved methods for the acquisition and interpretation of NMR metabolomic data. *Biochem. Biophys. Res. Commun.* 310:943–948.
- Wold, S., K. Esbensen, and P. Geladi. 1987. Principal component analysis. *Chemom. Intell. Lab. Syst.* 2:37–52.
- Xu, C., Z. Wang, G. W. Liu, X. B. Li, G. H. Xie, C. Xia, and H. Y. Zhang. 2008. Metabolic characteristic of the liver of dairy cows during ketosis based on comparative proteomics. *Asian-Australas. J. Anim. Sci.* 21:1003–1010.
- Yin, P., P. Mohemaiti, J. Chen, X. Zhao, X. Lu, A. Yimiti, H. Upur, and G. Xu. 2008. Serum metabolic profiling of abnormal savda by liquid chromatography/mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 871:322–327.
- Yudkoff, M., Y. Daikhin, I. Nissim, O. Horyn, A. Lazarow, B. Luhovyy, S. Wehrli, and I. Nissim. 2005. Response of brain amino acid metabolism to ketosis. *Neurochem. Int.* 47:119–128.