Pathway analysis of plasma different metabolites for dairy cow ketosis

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ABSTRACT

Metabolic profiling was performed using nuclear magnetic resonance (NMR)-based metabolomics to explore panoramic changes in ketosis metabolism in dairy cows, and to further understand changes in metabolites when ketosis occurred. According to clinical signs and plasma levels, 81 multiparous Holstein cows from a dairy farm were divided into clinical ketosis, subclinical ketosis and healthy control groups during 7–21 d after calving. Different metabolites were identified by NMR and combined with multivariate statistical analysis to determine the pathway. There were 29 metabolites obtained, including three carbohydrates, six lipids, 14 amino acids and six other metabolites. Consulting a database and the metabolite analysis showed that these metabolites were mainly related to amino acid, fat and carbohydrate metabolisms. The results indicated that metabolomics revealed changes in the occurrence and development of dairy cow ketosis, and also that the different metabolites could be potential markers and indicators to diagnose ketosis.

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Introduction

Dairy cow ketosis is a common nutrition metabolic disease caused by carbohydrate and fat metabolism disorder (Erb & Grohn 1988). Normally, the incidence of ketosis is observed from 2 to 6 parities in 1-month postpartum (Baird 1982). Ketosis is associated with a severe negative energy balance—insufficient energy intake cannot sustain the high-energy requirements for milk production during the early lactation period. To counteract this negative energy balance, fat from adipose tissue stores in cows has to be oxidised, thus large quantities of non-esterified fatty acids in the blood are oxidised to ketone bodies—acetoacetic acid, β-hydroxybutyric acid (BHBA) and acetone (Xu & Wang 2008). Furthermore, ketosis in dairy cows can decrease milk production and affect economic performance.

Energy metabolism, oxidative stress, clinical pathology and immune function have been examined during dairy cow ketosis. These previous studies showed that a variety of metabolic disorders or dysfunctions may occur during ketosis. However, panoramic evaluation of metabolism disorder during the process of ketosis remains ill-defined.

Metabolomics is an important branch of systems biology which studies endogenous metabolism stimulated by internal and external factors (Nicholson et al. 1999). Common metabolomic technologies include nuclear magnetic resonance (NMR), liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry. NMR combined with multivariate statistical analysis can analyse diseases to obtain new biomarkers and provide a new way for revealing pathogenesis and identity of diseases. Several metabolomic studies have been reported in humans and rats with metabolic diseases. Klein and Butchereit (2012) analysed milk using NMR, which indicated that the ratio of choline and choline phosphate could be used as a biomarker of dairy cow ketosis, but this was only preliminary study of milk metabolomics. The objective of the present study was to use 1H NMR metabolomics to study plasma metabolic profiling in dairy cows. We aimed to provide the foundation for revealing the mechanism of ketosis in dairy cows and determine new biomarkers of ketosis.

Materials and methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the
Ethics of Animal Experiments of the Heilongjiang Bayi Agricultural University (Permit no. 20120319-1). The surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimise suffering.

**Animals grouping and sample collection**

The experimental 81 multiparous Holstein cows were from a dairy cattle farm in Heilongjiang, China. They were selected randomly during 7–21 d postpartum, and all samples were collected during May–July. Cows were divided into three groups according to clinical symptoms and plasma BHBA concentration. There were 24 cows with clinical ketosis (CK) that had typical clinical signs such as loss of appetite, indigestion, anorexia, depression, low milk production and rapid consumption of body fat; these had plasma BHBA concentrations >1.60 mmol·L⁻¹. There were 33 cows in the subclinical ketosis (SK) group with few clinical signs and plasma BHBA concentrations >1.20 mmol·L⁻¹. There were 24 healthy control (C) cows with no clinical signs and normal plasma BHBA concentrations <1.00 mmol·L⁻¹ (Oetzel 2004). Experimental animals were fed in a freestall feeding system with a total mixed ration during the transition period, of the following composition: feed concentrate (8–9 kg), silage (17–20 kg), hay (3.5–4.0 kg) and fat (300–400 g). The diet nutrition levels are the following: dry matter (DM) 55.60%; crude protein 16%; net energy of lactation 4.18 × 10³ kJ·kg⁻¹ DM; fat 5.60%; neutral detergent fibre: 39.10%; acid detergent fibre 20.30%; calcium 180 g; and phosphorus 116 g.

After an overnight fast, venous blood samples (10 mL) were stabilised in sodium heparin in the morning and immediately centrifuged at 1400g for 10 min at room temperature. The supernatants were transferred and stored at −80 °C. Clinical parameters and plasma physics and chemistry indexes were recorded and analysed by one-way analysis of variance using SPSS (SPSS Inc., Chicago, IL). The blood samples were analysed using ELISA kits (Shanghai BlueGene Biotech Co. Ltd., Shanghai, China).

**1H NMR spectra of experimental samples**

Plasma samples of experimental animals were thawed at room temperature. Approximately 400 mL of plasma was mixed with 170 µL of deuterium oxide. The solution was centrifuged at 12,000g at 4 °C for 10 min. Aliquots (500 µL) of the supernatants were transferred to 5-mm NMR tubes.

Nuclear Overhauser effect spectroscopy impulse sequence was used in a 600-MHz NMR spectrometer, and time of flight was set to the position of the water peak. The flame ionisation detector signal was transferred into NMR profiling by 32 k Fourier. The collected data were transformed into one-dimensional data by call macroinstruction in variable nuclear magnetic resonance, and trisodium phosphate as the place of chemical shift reference peak. The 1H spectra were segmented and integrated according to default by the calling application of NMR. Integral data were stored as Excel files after normalisation for principal component analysis (PCA). Data were input in SIMCA-P + (V11.0 Umetrics AB, Umea, Sweden) for PCA and orthogonal partial least squares discriminant analysis (OPLS-DA). According to results of NMR spectograms, PCA and OPLS-DA, the plasma compositions in experimental and control groups were determined.

**Results and discussion**

**Clinical information**

The clinical parameters, including age, parity, postpartum days, body condition score (BCS), blood glucose and BHBA, are shown in Table 1. The BCS (a five-point scale) of 1 (thin) to 5 (obese) points with intervals of 0.25 was used to assess body fat stores (Ferguson et al., 1994). The milk yield of cows was determined on the same day as the collection of blood samples. The differences among the three groups in number of dairy cows, age, postpartum days, parity and milk yield, were not significant (p > 0.05). However, BHBA and glucose plasma levels were significantly different among the three groups (p < 0.05).

**Multivariate statistical analysis**

PCA and OPLS-DA results for plasma metabolic spectra of experimental cows are shown in Figures 1 and 2, respectively. PCA showed three groups of test samples but with a degree of overlap (Figure 1), and principal

| Parameter          | CK        | SK        | C         |
|--------------------|-----------|-----------|-----------|
| Number             | 24        | 33        | 24        |
| Postpartum days    | 13 ± 5    | 14 ± 4    | 16 ± 4    |
| Age, year          | 3 ± 2     | 3 ± 1     | 3 ± 2     |
| Parity             | 2 ± 1     | 2 ± 1     | 2 ± 1     |
| Body condition score| 3.05 ± 0.31A | 3.11 ± 0.35AB | 3.16 ± 0.37B |
| Glc, mmol L⁻¹      | 1.80 ± 0.71A | 2.72 ± 0.54B | 3.60 ± 0.36C |
| BHBA, mmol L⁻¹     | 2.61 ± 0.49A | 1.24 ± 0.34B | 0.74 ± 0.67C |

Different letters in the same row indicate a significant difference between the groups (p < 0.05); the same letter in the same row means no significant difference between groups (p > 0.05). CK: clinical ketosis group; SK: subclinical ketosis group; C: healthy control group; MY: milk yield; Glc: glucose; BHBA: β-hydroxybutyric acid.
component 1 (PC1) and PC2 represented 60.4 and 16.6% of variance, respectively. The OPLS-DA model of pattern recognition uses the first (t[1]P) and the second major component (t[2]O) (Figure 2(a–c)) and showed that the main metabolites differed between different groups.

**Amino acid metabolism**

The OPLS-DA results for different metabolites are shown in Table 2. All amino acids (mainly involved in the Krebs cycle), ketogenesis, fatty acid oxidation, were significantly lower in the CK than the SK group. The ketogenic amino acid lysine; the glucogenic amino acids histidine, glutamic acid and glutamine were progressively lower; 1-methyl histidine and 3-methyl histidine were lower in the CK compared with the SK and C groups. Additionally, 1-methylhistidine and 3-methylhistidine are isomerides. Levels of the glucogenic amino acids alanine, proline, glycine and valine were lower in the CK than the C group. Levels of leucine and isoleucine were higher in the SK than the other two groups. Levels of the glucogenic and ketogenic amino acids isoleucine, tyrosine and phenylalanine were lower in the CK than the C group. Levels of creatine, which mainly participates in fatty acid oxidation, were higher in the CK than the SK group.

According to different amino acids found in the present study and information on their metabolic pathways, an amino acid metabolic pathway network was constructed for occurrence of dairy cow ketosis (Figure 3). The main function of glycine and alanine is to produce glucose (Tamminga et al. 1997), and more sugar precursors are needed for energy supplementation when the body experiences a negative energy balance (Yudkoff et al. 2001). In this experiment, glycine levels were higher in the SK than the C group, indicating that protein mobilisation increased to provide glucose and amino acid supplements to meet the body’s energy requirements. The 1-methylhistidine and 3-methylhistidine are isomerides, and glutamate,

| No. | Metabolites | CK-C | SK-C | CK-SK |
|-----|-------------|------|------|-------|
| 1   | Glycine     |      | ↑    |       |
| 2   | Alanine     |      |      |       |
| 3   | 1-Methylhistidine |  |      |       |
| 4   | 3-Methylhistidine |  |      |       |
| 5   | Glutamate   |      |      |       |
| 6   | Glutamine   |      |      |       |
| 7   | Proline     |      |      |       |
| 8   | Valine      |      | ↑    |       |
| 9   | Leucine     |      |      |       |
| 10  | Lysine      |      |      |       |
| 11  | Isoleucine  |      |      |       |
| 12  | Tyrosine    |      |      |       |
| 13  | Phenylalanine |    |      |       |
| 14  | Creatine    |      |      |       |

' a' represents clinical ketosis group compared with the healthy control group (CK-C); ' b' represents the subclinical ketosis group compared with the healthy control group (SK-C); ' c' represents the clinical ketosis group compared with the subclinical ketosis group (CK-SK). ' † † ' represents the former content higher in the plasma compared with the latter; ' † † † ' represents the former content lower in plasma compared with the latter; ' † ' represents content in plasma with no significant difference ($p < 0.05$).

Figure 1. The plot of PCA scores of plasma samples obtained from the clinical ketosis (CK), subclinical ketosis (SK) and control (C) groups without outliers. On the scores plot, each point represents an individual sample. The centre and margin of the ellipse indicate mean and standard deviation, respectively.

Figure 2. (a) Plot of scores of OPLS-DA models from the clinical ketosis (CK) and control (C) groups. (b) Score plot of OPLS-DA models from the subclinical ketosis (SK) and C groups. (c) Score plot of OPLS-DA models from the CK and SK groups.
glutamine, proline and valine are glucogenic amino acids. When ketosis occurred, more amino acids were needed for energy supplement, and mainly participated in the Krebs cycle. Amino acid contents were lower in the CK and SK than the C group, because the negative energy balance was more serious in the CK than the SK group and required more glucogenic amino acids for energy supplementation. Leucine and lysine are ketogenic amino acids, isoleucine can be either a glucogenic or a ketogenic amino acid, and their contents were lower in the CK than the SK group, because the body’s protein metabolism was strengthened when clinical ketosis occurred – more ketogenic amino acids resulted in ketogenesis, and hence elevated the levels of ketones. Contents of leucine and isoleucine were higher in the SK than in the C group, which may be a useful measure to determine subclinical ketosis following further research. Leucine and phenylalanine are compatible glucogenic and ketogenic amino acids, and their contents were lower in the CK than in the SK group, indicating that the body had a serious negative energy balance and needed more compatible amino acids for glucose. However, the Krebs cycle pathway was blocked resulting in more compatible amino acids entering the ketogenic path, and thus in elevated levels of plasma ketones.

Studies have shown that creatine supplied to rats is transferred to phosphocreatine in the body (Francaux & Demeure 2000). This produces ATP during movement, providing the energy the body requires, reducing the use of glycogen in vivo, decreasing levels of lactate and easing the negative energy balance. Our experimental results showed that the levels of creatine significantly increased only in the SK group, indicating that more creatine was needed to ease the serious negative energy balance. Further verification may confirm this as a specific diagnostic marker for subclinical ketosis.

**Glucose metabolites**

The concentrations of α-glucose, β-glucose and lactate gradually declined with the increasing severity of ketosis (Table 3). Both α-glucose and β-glucose are isomers, and participated in glycolysis/gluconeogenesis; and lactate is an end-product of glycolysis and precursor of gluconeogenesis. The SK cows were experiencing a severe negative energy balance. Even if the milk production of the cows was low the energy offered with the diet appeared high. The glucose levels in SK were very low, and it is probable that feed intake was also very low.

The lactation peak occurs earlier than the feeding peak, and a lot of blood glucose is lost for synthesis of lactose, with the body supplying glucose through promoting lactic acid and other glucogenic substances by neuroendocrine regulation (Xu et al. 2008). However, in this experiment, plasma glucose and lactate both
decreased (Table 3), indicating that gluconeogenesis could not maintain normal glucose levels and resulted in a negative energy balance. To maintain milk and glucose, fat mobilisation was increased and fatty acid oxidative metabolism was enhanced at the same time as ketones increased (Figure 4).

**Lipid metabolites**

Concentration of ketone bodies in plasma was higher with the increased severity of ketosis as an outcome of incomplete fatty acid oxidation (Table 4). Levels of choline, which mainly participates in lipid transfer, decreased in the CK and did not change in the SK group. Inositol mainly participates in phosphoinositide metabolism, and the contents increased in the CK and decreased in the SK group.

Low-density lipoprotein (LDL) is combined with its receptor in the body and enters cells to obtain lipids, cholesteryl ester is hydrolysed to free cholesterol and fatty acids by the action of lysosome. Acetyl coenzyme A (acetyl-CoA) is obtained after further fatty acid oxidation, and supplies energy through the Krebs cycle. When the Krebs cycle is hampered, excessive acetyl-CoA can generate ketones. When dairy cow ketosis occurs, body fat mobilisation is needed to supply energy to counteract the negative energy balance, which results in decreased contents of LDL and very low-density lipoprotein (VLDL) (Klein & Buttchereit 2012), consistent with results in the present study.

Choline can accelerate liver triglyceride transfer and prevent its abnormal accumulation in the liver (Eagle et al. 1957), promote dry matter intake and ease any negative energy balance (Van Soest 1994). Therefore, choline contents were higher in the SK than the other groups, indicating that high choline benefitted the energy metabolism when subclinical ketosis occurred and restrained the development of ketosis. Inositol can generate inositol ester by combining with fatty acids and phosphoric acid, and can promote fat metabolism and reduce blood fat (Ireland et al. 2009). In this experiment, the inositol levels increased in the CK and decreased in the SK group, indicating that severity of ketosis may be related to inositol use, but this requires further research.

**Other metabolites**

Citrate, which mainly participates in the Krebs cycle, showed decreased levels in the CK and no obvious change in the SK group (Table 5). In the current study, formate was lower in the CK than in the SK and C groups, and there was no obvious change in the SK group – formate mainly participates in pyruvate metabolism and is a product of pyruvate. Acetate mainly participates in the Krebs cycle and increased in the CK and SK compared with the C group, with no significant differences between the two types of ketosis. Allantoin mainly participates in purine metabolism and increased only in the SK group. The metabolic pathways corresponding to unassigned resonances 1 and 2 were unknown, but the levels of unassigned resonance 1 increased with the severity of ketosis, similar to the changes for ketones. Levels of unassigned resonance 2 increased in the CK compared with the

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**Table 4. Lipid metabolites with differential expression among groups.**

| No. | Metabolites | CK-C | SK-C | CK-SK | Metabolic pathway |
|-----|-------------|------|------|-------|------------------|
| 1   | Choline     | –    | –    | ↑     | Fat, cholesterol metabolism |
| 2   | VLDL        | –    | –    | ↑     | Lipid transfer |
| 3   | LDL         | ↓    | –    | ↑     | Lipid transfer |
| 4   | Myo-inositol| ↓    | ↑    | –     | Inositol phosphate metabolism |
| 5   | Acetone     | ↑    | ↑    | –     | Fatty acid oxidation |
| 6   | BHBA        | ↑    | ↑    | –     | Fatty acid oxidation |

‘a’ represents clinical ketosis group compared with the healthy control group; ‘b’ represents the subclinical ketosis group compared with the healthy control group; ‘c’ represents clinical ketosis group compared with the subclinical ketosis group; ‘↑’ represents the former content higher in plasma compared with the latter; ‘↓’ represents the former content lower in plasma compared with the latter; ‘–’ represents content in plasma with no significant difference. LDL: low-density lipoprotein; VLDL: low-density lipoprotein; BHBA: β-hydroxybutyric acid.
Table 5. Other metabolites of differential expression among groups.

| No. | Metabolites | CK-Ca | SK-Cb | CK-SKc | Metabolic pathway |
|-----|-------------|-------|-------|--------|-------------------|
| 1   | Citrate     | ↓     | –     | ↓      | Tricarboxylic acid cycle |
| 2   | Formate     | ↓     | –     | ↓      | Pyruvate metabolism   |
| 3   | Acetate     | ↑     | ↑     | –      | Tricarboxylic acid cycle |
| 4   | Allantoin   | –     | –     | ↑      | Purine metabolism     |
| 5   | Unassigned  | ↑     | ↑     | ↑      | Unassigned resonance 1 |
| 6   | Unassigned  | –     | –     | ↓      | Unassigned resonance 2 |

a’ represents clinical ketosis group compared with the healthy control group; b’ represents the subclinical ketosis group compared with the healthy control group; c’ represents clinical ketosis group compared with the subclinical ketosis group; ′↑′ represents the former content higher in plasma compared with the latter; ′↓′ represents the former content lower in plasma compared with the latter; ′-′ represents content in plasma with no significant difference.

...SK group. However, these unassigned resonances require further identification.

The experimental results showed some other metabolic substance abnormalities such as citric, acetic and formic acids. As an intermediate of the Krebs cycle, citric acid is a synthetic product of oxaloacetic acid and acetyl-CoA. Therefore, decreases in citric acid are related to decreases in oxaloacetic acid, as excess acetyl-CoA of fatty acid decomposition is blocked in the Krebs cycle due to decreases in citric acid and so proved energy to synthesise ketones. However, the limited ability of the body to use ketones results in accumulation of ketones in the blood and so in ketosis.

As a decomposition product of non-esterified fatty acid (NEFA), the main function of acetic acid is to be activated as acetyl-CoA for energy or be transformed acid were higher in both the CK and SK than in the C group, but there was no significant difference between two ketosis groups. This indicated that lipid mobilisation and NEFA decomposition were improved when ketosis occurred, contents of acetic acid were increased and this would maintain butter-fat percentage and promote ketosis.

According to metabolic pathways, formic acid is the product of pyruvic acid. Contents of formic acid in the CK group decreased significantly, while those in the SK group showed no significant change. This experiment indicated that the formate generated by pyruvic acid decreased and instead the pyruvic acid generated acetyl-CoA, which resulted in ketogenesis and elevated blood ketone content.

Uricase can resolve uric acid, an end-product of purine metabolism in mammals, into allantoin. Research has shown that uric acid is associated with triglyceride metabolism. More uric acid restrains enzymes that catalyse decomposition of triglycerides. In this experiment, contents of allantoin were higher in the SK group, and uric acid can decrease the metabolism of triglyceride and increase decomposition of triglycerides. Whether this is related to occurrence of ketosis in dairy cows will require further research.

Conclusions

Different metabolites known to be associated with ketosis were investigated in plasma of dairy cows using 1H NMR-based metabolomics, a powerful technology to obtain markers of metabolic disease, and multivariate statistical analysis. It was also revealed that the change and role of different metabolites in dairy cow ketosis provides a new basis for understanding the pathogenesis of ketosis and lays a foundation for its control in the future.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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