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Abstract

Ketosis is associated with high milk yield during lactating or insufficient feed intake in lactating dairy cows. However, few studies have been conducted on the metabolomics of ketosis in Korean lactating dairy cows. The present study aimed to investigate the serum and urine metabolites profiling of lactating dairy cows through proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy and comparing those between healthy control (CON) and subclinical ketosis (SCK) groups. Six lactating dairy cows were categorized into CON and SCK groups. All experimental Holstein cows were fed total mixed ration. Serum and urine samples were collected from the jugular vein of the neck and by hand sweeping the perineum, respectively. The metabolites in the serum and urine were determined using ^{1}H-NMR spectroscopy. Identification and quantification of metabolites was performed by Chenomx NMR Suite 8.4 software. Metabolites statistical analysis was performed by Metaboanalyst version 5.0 program. In the serum, the acetoacetate level was significantly (p-value < 0.05) higher in the SCK group than in the CON group, and whereas acetate, galactose, and pyruvate levels tended to be higher. CON group had significantly (p-value < 0.05) higher levels of 5-aminolevulinate and betaine. Indole-3-acetate, theophylline, p-cresol, 3-hydroxymandelate, gentisate, *N*-acetylglucosamine, *N*-nitrosodimethylamine, xanthine, and pyridoxine levels were significantly (p-value < 0.05) higher in the urine of the SCK group than that in the CON group, which had higher levels of homogentisate, ribose, gluconate, ethylene glycol, maltose, 3-methyl-2-oxovalerate, and glycocholate. Some significantly (p-value < 0.05) different metabolites in the serum and urine were associated with ketosis diseases, inflammation, energy balance and body weight. This study will be contributed useful a future ketosis metabolomics studies in Korea.

Keywords (3 to 6): Metabolites; Subclinical ketosis; Lactating dairy cow; Serum; Urine; Proton nuclear magnetic resonance spectroscopy

Introduction

Ketosis is common metabolic disease of dairy animals, and is associated with high milk yield during lactation or inadequate feed intake for energy that results in negative energy balance (NEB) [1]. Ketosis in lactating dairy cow decreases milk production and has negative effects on reproductive capacity [2]. In addition, such cows have a higher risk of developing periparturient disease such as lameness, mastitis, metritis, and retained placenta [3-6]. Ketosis is associated with an increased concentration of ketone bodies (acetoacetate, acetone and beta-hydroxybutyrate [BHB]) in the biofluid (milk, plasma, serum and urine) [7]. The underlying cause of the high concentration of ketone body metabolites are low blood glucose levels associated with hypoinsulinemia, which results in the mobilization of fatty acid (FA)s generated from adipose tissue [7]. Ketosis is classified into subclinical ketosis (SCK), clinical ketosis (CK), and type I and II ketosis. In SCK and CK, BHB concentration ranges from 1.2 to 1.4 mM/L and 2.6 to 3.0 mM/L, respectively [8,9]. Type I ketosis occurs between 3 and 6 weeks postpartum when the energy requirement for milk production is the highest [10]. Type II ketosis can lead to complications such as fatty liver via an increase in blood glucose and insulin levels because of excessive feed intake during the dry period [7,10]. Ketosis leads to considerable economic loss in the dairy industry [11]; therefore, research on the prevention and diagnosis of ketosis is required.

Metabolomics studies pertaining to ruminant biofluids including milk, plasma, rumen fluid, serum, and urine are performed using many metabolite analyzers (nuclear magnetic resonance [NMR] spectroscopy, gas chromatography-mass spectrometry [GC-MS], liquid chromatography-mass spectrometry [LC-MS], etc.). Moreover, metabolomics studies pertaining to metabolic diseases (acidosis, ketosis etc.) have been conducted. Ketosis research using metabolites in the serum and plasma and the comparative study of metabolites in the plasma of CK, SCK, and healthy lactating dairy cows have identified several metabolic pathways associated with CK and SCK by GC-MS, NMR, and LC-MS [11-13]. In another study, plasma metabolite profiling was performed for type I and II ketosis by proton NMR (^{1}H-NMR) spectroscopy [14]. Another study compared the urine metabolites in healthy and SCK cows by NMR, direct injection GC-MS and LC-MS/MS [15]. However, there have been fewer urine-based research on ketosis in lactating dairy cows compared with those on other biofluids such as serum, plasma, and milk. Metabolic profiling analysis of urine samples has identified biomarkers in various studies on humans [16,17]. Zhang et al. [15] reported a lacked of urinary metabolomics profiling for the identification of predictive biomarkers of ketosis in dairy cows. Therefore, it is necessary to study the urine metabolites of ketosis-induced dairy cows. Such studies will be useful in the future to search for biomarker candidates for ketosis diagnosis using blood (serum and plasma) and urine.

Recently, a metabolomics study on Hanwoo cattle and Holstein cows biofluids was performed by ^{1}H-NMR spectroscopy in Korea [18-20]. However, there have been few metabolomics studies on lactating dairy cow biofluids associated with ketosis Korea compared with those in other countries. In addition, in

the Korea, the Holstein species are representing lactating dairy cow, and most important ruminant breeds in the Korean dairy industry [21]. Therefore, it is essential to conduct metabolomics studies that can help us to identify metabolic biomarkers biofluids that can diagnosis ketosis in the future.

We hypothesized that the serum and urine metabolites profiling of lactating dairy cows would be different between healthy control (CON) and SCK groups. To test this hypothesis, we aimed to investigate the serum and urine metabolites profiling of lactating dairy cows using ^{1}H-NMR spectroscopy and compare between two groups. This metabolic study will be helpful for developing strategies to reduce lactating dairy cow ketosis in Korea.

Materials and Methods

Animals and sampling

Healthy and SCK Holstein lactating dairy cow general information (month-old, body weight, parity, milk yield, BHB concentration and number of experimental animals), total experimental day (14 days), diet adaptation period (4 days) blood BHB concentration monitoring period (9 day), blood and urine samples collected day (last day of the experiment) were same of previously Eom et al. study [22].

All experimental cows were fed total mixed ration (TMR). The consumed CON and SCK groups feed intake amount, same of previously Eom et al study [22]. The results of chemical composition of the TMR is shown Table 1. Contents of acid detergent fiber, calcium, crude protein, dry matter, neutral detergent fiber and phosphorus in TMR was determined as described by Association of Official Analytical Communities [23,24] and Van Soest et al. [25] methods.

Blood samples collected and storage methods until ^{1}H-NMR spectroscopy analysis was determined as described by Kim et al. [19] and Eom et al. [20] methods. Urine samples collected and storage methods until ^{1}H-NMR spectroscopy analysis was determined as described by Kim et al. [19] and Eom et al. [20] methods.

Prepared ^{1}H-NMR spectroscopy analyses

The ^{1}H-NMR spectroscopy analysis of serum and urine samples was performed by following methods modified from sun et al. [26], Jung et al. [27], Bertram et al. [28] and Jeong et al. [29] methods.

The spectra of two samples were obtained on a SPE-800 MHz NMR-MS Spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) at 298 K using a 5 mm triple-resonance inverse cryoprobe with Z-gradients (Bruker BioSpin CO., Billerica, Massachusetts, USA) and method condition described by Kim et al. method [30].

Metabolites measurement, quantification, and statistical analysis

Serum and urine metabolites identification and quantification methods and data collected was performed by following methods modified from Kim et al. [19] and Eom et al. [20] methods. Metabolites data statistical analyses were using the Metaboanalyst version 5.0 program (http://www.metaboanalyst.ca), an open source *R*-based program for metabolomics. For the serum and urine metabolites analysis, when 50% of samples were under the identification limit or had at least 50% of missing values, they were eliminated from the analysis. The missing values were replaced by a value one-half of the minimum positive value from the original data. In addition, statistical analyses methods were determined as described by Kim et al. [19] and Eom et al. [20] methods. Univariate Student's *t*-test were used to quantify difference between metabolite profiles of the serum and urine samples. In addition, principal component analysis (PCA), partial least square-discriminant analysis (PLS-DA), variable importance in projection (VIP) scores and metabolic pathway results were determined as described by Kim et al. [19] and Eom et al. [20] methods.

Results

Multivariate data analysis

To analyze the variations in the serum and urine metabolites profiling of CON and SCK groups, we performed PCA and PLS-DA. In the serum PCA score plot (Fig 1A), two groups were not separated (PC 1 : 30.2%; PC 2 : 24%). In the urine PCA score plots (Fig 1B) two groups were not separated (PC 1 : 31%; PC 2 : 24.5%).

The serum PLS-DA score plots (Fig 2A), for the two groups were clearly separated (component 1 : 24.6%; component 2 : 21.6%). The urine PLS-DA score plots (Fig 2B) for two groups were clearly separated (component 1 : 28.1%; component 2 : 23.3%). These results show different in the concentration of serum and urine metabolites between CON and SCK groups.

Detected and quantification of serum and urine metabolites

Suppl Tables 1-7 and, Suppl Figs 1 and 2 summarize the detected and quantified metabolites in the two groups. In the CON group, 98 metabolites were detected and divided into 12 chemical classes in the serum. In addition, a total of 52 metabolites were quantified. In the SCK group, 83 metabolites were detected and divided into 12 chemical classes in the serum. In addition, a total of 55 metabolites were quantified.

In the CON group, 144 metabolites were detected and divided into 13 chemical classes in the urine. In addition, a total of 93 metabolites were quantified. In the SCK group, 168 metabolites were detected and divided into 14 chemical classes in the urine. In addition, a total of 93 metabolites were quantified.

Differences in serum and urine metabolites between healthy and subclinical ketosis groups

Table 2 shows the significant trends (p-value < 0.05) and tendencies (0.05 ≤ p-value < 0.1) of different metabolites in the serum and urine of the two groups. Acetoacetate and succinate levels were significantly higher, whereas acetate, galactose, and pyruvate levels tended to be higher but non-significantly in the serum of the SCK group than in the CON group. In contrast, 5-aminolevulinate (5-ALA) and betaine levels were significantly higher, whereas lactulose and 3-methylxanthine levels tended to be higher in the CON group than in the SCK group.

Indole-3-acetate, theophylline, p-cresol, 3-hydroxymandelate, gentisate, *N*-acetylglucosamine, *N*-nitrosodimethylamine, xanthine, and pyridoxine levels in the urine of the SCK group were significantly higher, and nicotinurate, acetoin, trimethylamine *N*-oxide, 3-methylxanthine (3-MX), indole-3-lactate, carnosine, and BHB levels tended to be higher compared with those in the CON group. In contrast, homogentisate, ribose, gluconate, ethylene glycol, maltose, 3-methyl-2-oxovalerate, and glycocholate levels were significantly higher, and alanine level tended to be higher in the SCK group than in the CON group.

As shown in Fig 3A and B, the evaluation of over to 1.5 VIP score of PLS-DA showed 15 and 18 metabolites between the two groups of serum and urine, respectively. Acetoacetate, pyruvate, and O-acetylcholine were higher VIP scores in the serum of the SCK group compared with those in the CON group. In contrast, 3-methylxanthine, syrinagate, and arginine were higher VIP scores in the serum of the CON group compared with those in the SCK group. Theophylline, gentisate, and *N*-nitrosodimethylamine were higher VIP scores in the urine of the SCK group compared with those in the CON group. In contrast, ethylene glycol, gluconate, and maltose were higher VIP scores in the urine of the CON group compared with those in the SCK group.

Metabolic pathway analysis

In the serum profiling of including porphyrin and chlorophyll; glycine, serine and threonine; citrate cycle; and alanine, aspartate, and glutamate, four metabolic pathways significantly (p-value < 0.05) differed between the two groups. The tendency of the following four metabolic pathways tendency (0.05 ≤ p-value < 0.1) differed in the serum between the two groups; such as tyrosine metabolism; butanoate metabolism; synthesis and degradation of ketone bodies; and valine, leucine and isoleucine degradation (Table 3 and Fig 4A).

In the urine, the following 11 metabolic pathways significantly (p-value < 0.05) differed between the two groups; such as ubiquinone and other terpenoid-quinone biosynthesis; alanine, aspartate and glutamate metabolism; selenocompound metabolism; aminoacyl-tRNA biosynthesis; valine, leucine and isoleucine degradation; valine, leucine and isoleucine biosynthesis; pentose phosphate pathway; tyrosine metabolism; starch and sucrose metabolism; primary bile and biosynthesis; and tryptophan metabolism (Table 4 and Fig 4B).

Discussion

In lactating dairy cows with NEB, the increased glycine concentration in plasma is related to the breakdown of muscle protein [31] or to the de novo synthesis of glycine from threonine and serine [32]. Shibano et al. [33] reported that glycine in the serum could be used as a marker for EB and metabolic position in lactating dairy cows. In addition, the ratio of glycine to alanine concentration in the serum was used as a biomarker for malnutrition in lactating dairy cows during early lactation [33]. In this study, glycine concentration was higher in the SCK group. Alanine concentration was higher in the CON group; however, not significance different (p-value > 0.05). High concentration of glycine, kynurenine, and pantothenate and low concentration of arginine as a novel biomarker for NEB diagnosis [34]. In addition, the low concentration of arginine in lactating dairy cows with NEB induces an increase in nitrogen oxide concentration with an attendant increase in blood flow, which is useful for higher nutrients supply for milk production in the mammary gland [35]. In this study, pantothenate concentration was higher in the SCK group; however, not significance different (p-value > 0.05). Arginine was quantified only CON group and the VIP score was high in the CON group. The substrates for de novo synthesis of FA are BHB and acetate and are used by the mammary epithelial cells to synthesize short and medium chain FAs and sixteen-carbon FAs [36]. During the NEB period, the de novo synthesis of FA decreases and the body commence to use its own storage of energy [37]. In addition, type I and II ketosis have higher concentrations of acetate and BHB [14]. In this study, BHB concentration was higher in the SCK group; however, not significance different (p-value > 0.05), and the acetate concentration in the SCK group was tended to be higher ($0.05 \leq p$-value < 0.1) compared with the CON group. Low concentrations of blood glucose are related to hypoinsulinemia, which subsequently activates FA mobilization from tissues, thereby increasing the concentration of ketone body metabolites [7]. Carocho et al. [38] reported that the caloric of 100 g glucose and sucrose led to different peaks in blood glucose concentration. Sucrose is a disaccharide, comprising one molecule of glucose and one molecule of fructose [39]. Therefore, sucrose is connected to the concentration of glucose in the blood. In this study, glucose concentration was higher in the CON group; however, the difference was not significant (p-value > 0.05). Sucrose was quantified in the CON group but not in the SCK group, and the VIP score was also higher in the CON group. Blood BHB, acetoacetate, and acetone are associated with the incomplete beta-oxidation of mobilized excess of

fats that, result in ketosis [40,41]. Among them, BHB and acetone concentrations in the plasma are useful in the diagnosis of SCK in lactating dairy cows during early lactation [42,43]. In this study, BHB and acetone concentrations were higher in the SCK group; however, the difference was not significant (p-value > 0.05). Acetoacetate concentration was significantly (p-value < 0.05; VIP score : 1.85) higher in the SCK group.

5-ALA is a dietary supplement for livestock that can affect the synthesis of heme and positively influence the iron status of hemoglobin in animals [44]. In addition, 5-ALA supplementation improved milk protein, fat and casein in dairy cows [45,46]. 5-ALA in blood is a product of condensing succinyl-CoA and glycine through the catalytic activity of 5-ALA synthase [47]. Hendawy et al. [48] reviewed biological activities of 5-ALA and reported, for example, antioxidant, anti-inflammatory, and immunomodulator activities. NF-κβ induces a variety of genes that encode proteins involved in inflammation including TNF, IL-1, and interleukin-23 [49]. Betaine supplementation improved milk yield, fat and FA synthesis [50]. Betaine in serum has shown several anti-inflammatory effects including the inhibition of NF-κβ [49]. Ametaj et al. [51] reported a potential role of immune factors in triggering systemic inflammation during the transition period in the pathobiology of metabolic disorders (e.g, concurrent disease with type II ketosis). In this study, 5-ALA (p-value < 0.01; VIP score : 1.67) and betaine (p-value < 0.05; VIP score : 1.60) concentration were significantly lower in the SCK group. Therefore, 5-ALA and betaine levels in the serum of lactating dairy cows are potential biomarkers for the diagnosis of ketosis. However, further research is needed on the relationship between the two metabolite (5-ALA and betaine) and ketosis diagnosis.

SCK involves an increase in the levels of ketone body metabolites in the urine, and the distinct signs of CK disease are absent [7]. Recently, the acetoacetate level in urine was used as a biomarker for ketosis diagnosis in the dairy industry [15]. This method is a quantitative test limited by its short sensitivity, and is used only for examine purposes [52]. In this study, acetoacetate and acetone concentrations were higher in the SCK group; however, the difference was not significantly (p-value > 0.05). The BHB concentration tended to be higher ($0.05 \leq p$-value < 0.1), as did the VIP score (1.85) was also higher in the SCK group. Kawasaki et al. [53] reported that urine fructose concentration decreases during ketosis in patients. Acute administration of fructose promotes other adverse metabolic diseases, including hyperuricemia and lactic acidosis [54]. Therefore, the fructose level in the urine of lactating dairy cows might be considered a potential biomarker for the diagnosis of ketosis. In this study, fructose concentration was higher in the SCK group; however, the difference was not significantly (p-value > 0.05). Amino acids are substantial precursors for associated with gluconeogenesis and ketogenesis [55,56], and crucial moderator or intervening in diverse metabolic pathways, including cell signaling, immunity, growth, maintenance, and oxidative stress [57-59]. Therefore, amino acid metabolism is essential for sustained condition and for preventing diseases (metabolic and contagious) [15]. Pantothenate has importance metabolites in production of carbohydrate and FA metabolism associated with energy [60]. Zhang et al. [15] reported

that amino acid metabolites (arginine, aspartic acid, glutamate, glycine, alanine, cysteine, isoleucine, lysine, phenylalanine, and tyrosine) and carnosine, *N*-acetylglutamate, 1-methylhistidine, 3-methylhistidine and pantothenate were higher concentration in the urine of normal cow. In this study, glycine, methylhistidine, and pantothenate concentrations were higher in the CON group; however, the difference was not significantly (p-value > 0.05). Alanine concentration tended to be higher ($0.05 \leq p$-value < 0.1) in the CON group. In addition, aspartate and glutamate metabolites was not significance different (p-value > 0.05) between the two groups.

Homogentisate in urine is produced through the catabolism of phenylalanine by homogentisate 1,2 dioxygenase (HGD) [61], and HGD affects body weight and sirloin cross-sectional area in cattle [62]. Holtenius and Holtenius [10] reported that CK in lactating dairy cows reduced milk yield and body weight. In this study, homogentisate concentration was significantly (p-value < 0.01; VIP score : 1.81) higher in the CON group. Theophylline is a xanthine-based metabolite and, an intermediate product in the metabolic process of caffeine and 3-MX. This metabolite can be excreted through the kidneys and has a diuretic effect [63,64] and can cause a variety of side effects in cows, such as acid-base and electrolyte imbalances [65]. In this study, theophylline concentration was significantly (p-value < 0.01; VIP score : 2.01) higher and 3-MX concentration was tended to be higher ($0.05 \leq p$-value < 0.1; VIP score : 1.71) in the SCK group. The p-cresol concentration in urine may reflect the intake of dietary phenylalanine and tyrosine in ruminants, and thus may be a proxy of the overall N intake [66]. However, approximately half of the tyrosine content of rumen-administered casein was excreted as p-cresol [67]. Therefore, an excessively high concentration in the urine may have a negative effect on nitrogen metabolism in ruminants. In this study, p-cresol concentration was significantly (p-value < 0.05) higher in the CON group. Since the research on urine metabolites related to ketosis in lactating dairy cow is insufficient the relationship between metabolites and ketosis shown in this study will be helpful for minimizing the incidence of the disease. In addition, homogentisate, theophylline, 3-MX and p-cresol levels in the urine of lactating dairy cows are potential biomarkers for the diagnosis of ketosis. However, further research is needed on the relationship between the four metabolite (homogentisate, theophylline, 3-MX and p-cresol) and ketosis diagnosis.

The metabolites profiling of CON and SCK group lactating dairy cows were investigated by ^{1}H-NMR spectroscopy. In the serum, associated with inflammation (5-ALA and betaine) and positive energy balance (arginine) metabolites was high concentration in the CON group, whereas ketone bodies including acetoacetate and acetate were high concentration in the SCK group. In the urine, associated with gluconeogenesis (amino acids; alanine) and body weight (homogentisate) were high concentration in the CON group, whereas ketone bodies including BHB was high concentration in SCK group. In Korea, studies on metabolic profiling by ^{1}H-NMR spectroscopy are inadequate. Therefore, this study will contribute to future ketosis metabolomics studies in Korea by serving as a reference guide.

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Tables

Table 1. Ingredients and nutrients of the experimental diets

Items	Value (% of DM basis)
Ingredients, % of DM	
Concentrate	15.3
Soybean meal	2.40
Corn silage	47.2
Alfalfa hay	7.10
Tall fescue	9.40
Timothy	5.90
Energy booster[1)]	7.10
Cash Gold[1)]	4.50
Lyzin-Plus[2)]	0.20
Limestone[3)]	0.20
Zin Care[1)]	0.10
Supex-F[1)]	0.50
Trace minerals[4)]	0.05
Vitamins premix[5)]	0.05
Chemical composition (% of DM basis)	
Dry matter (DM), %	53.2
Crude protein	10.0
Neutral detergent fiber	28.2
Acid detergent fiber	16.9
Calcium	0.40
Phosphorus	0.15

[1)] Cofavet, Cheonan, Korea. Zin Care, Contained 16 GDU/g protease bromelain, 2.0×10^8 cfu/g. Supex-F, Contained 99% protected fat from of palm oil.

[2)] A.N.Tech, Cheonan, Korea. Lyzin-Plus, Contained 6.0% Zn, 0.9% Cu, 1.4% Mn, 5.0% chelated glycine.

[3)] Sungshin minefield, Jeongseon, Korea.

[4)] Trace minerals, Contained 0.40% Mg, 0.20% K, 4.00% S, 0.08% Na, 0.03% Cl, 400 mg of Fe/kg, 60,042 mg of Zn/kg, 16,125 mg of Cu/kg, and 42,375 mg of Mn/kg.

[5)] Vitamins premix, Provided approximately 5,000 KIU of vitamin A/kg, 1,000 KIU of vitamin D/kg, 33,500 mg of vitamin E/kg, and 2,400 mg of vitamin C/kg.

DM, dry matter.

Table 2. Differential enrichment of metabolites content of serum and urine between healthy and subclinical ketosis group

Metabolites	Classification	CON/ SCK[1)]	*p*-value	VIP score[2)]	FC[3)]
Serum					
5-aminolevulinate	Carboxylic acid	CON	6.21×10^{-3}	1.67	0.42
Betaine	Other	CON	1.46×10^{-2}	1.60	0.40
Acetoacetate	Carbohydrate	SCK	2.44×10^{-2}	1.85	-0.51
Succinate	Carbohydrate	SCK	3.45×10^{-2}	1.63	-0.42
Lactulose	Carbohydrate	CON	5.04×10^{-2}	0.94	0.16
Acetate	Carbohydrate	SCK	6.98×10^{-2}	0.79	-0.11
3-methylxanthine	Other	CON	7.63×10^{-2}	1.82	0.47
Galactose	Carbohydrate	SCK	8.24×10^{-2}	1.58	-0.55
Pyruvate	Carbohydrate	SCK	7.86×10^{-2}	1.80	-0.49
Urine					
Indole-3-acetate	Other	SCK	9.34×10^{-4}	1.36	-0.39
Homogentisate	Benzoic acid	CON	2.23×10^{-3}	1.81	0.69
Ribose	Carbohydrate	CON	2.50×10^{-3}	1.88	0.76
Gluconate	Organic acid	CON	5.97×10^{-3}	2.06	0.97
Theophylline	Other	SCK	5.99×10^{-3}	2.01	-0.96
p-cresol	Benzoic acid	SCK	1.11×10^{-2}	1.13	-0.29
Ethylene glycol	Lipid	CON	1.56×10^{-2}	2.55	1.67
Maltose	Carbohydrate	CON	2.37×10^{-2}	2.04	1.11
3-hydroxymandelate	Benzoic acid	SCK	2.72×10^{-2}	1.73	-0.82
Gentisate	Benzoic acid	SCK	2.96×10^{-2}	1.94	-0.80
N-acetylglucosamine	Carbohydrate	SCK	3.31×10^{-2}	1.63	-0.72
3-methyl-2-oxovalerate	Carboxylic acid	CON	3.65×10^{-2}	1.33	0.39
N-nitrosodimethylamine	Organic acid	SCK	4.19×10^{-2}	1.84	-0.74
Glycocholate	Lipid	CON	4.22×10^{-2}	1.82	0.76
Xanthine	Nucleoside, Nucleotide	SCK	4.25×10^{-2}	0.90	-0.20
Pyridoxine	Other	SCK	4.81×10^{-2}	1.73	-0.89
Nicotinurate	Organic acid	SCK	5.17×10^{-2}	1.43	-0.47
Acetoin	Other	SCK	5.35×10^{-2}	1.58	-0.77
Alanine	Amino acid	CON	6.71×10^{-2}	0.70	0.13
Trimethylamine *N*-oxide	Aliphatic acylic compound	SCK	6.97×10^{-2}	1.11	-0.35
3-methylxanthine	Other	SCK	7.00×10^{-2}	1.71	-0.96
Indole-3-lactate	Other	SCK	8.18×10^{-2}	1.34	-0.43
Carnosine	Amine	SCK	8.20×10^{-2}	1.21	-0.39
3-hydroxybutyrate	Lipid	SCK	9.03×10^{-2}	1.54	-0.85

[1)] CON/SCK, Comparison between healthy (CON) and subclinical ketosis (SCK) group.

[2)] VIP, Variable importance in the projection obtained from partial least square-discriminant analysis model.

[3)] FC, Fold change; Calculated as binary logarithm average concentration response ratio between CON and SCK group, where the positive value means that average concentration response of the metabolites in the former is larger than that in the latter and vice versa.

Table 3. Pathway analysis of significantly different serum metabolites compared with healthy and subclinical ketosis group

Metabolic Pathway	Total Cmpd[1)]	Hits[2)]	*p*-value	-Log (*p*-value)	FDR[3)]	Impact[4)]
Porphyrin and chlorophyll metabolism	30	1	6.55×10^{-3}	2.18	6.06×10^{-2}	0.03
Glycine, serine and threonine metabolism	34	3	7.57×10^{-3}	2.12	6.06×10^{-2}	0.05
Citrate cycle [tricarboxylic acid (TCA) cycle]	20	2	3.98×10^{-2}	1.40	0.15	0.08
Alanine, aspartate and glutamate metabolism	28	2	3.98×10^{-2}	1.40	0.15	0.00
Tyrosine metabolism	42	2	6.64×10^{-2}	1.18	0.15	0.00
Butanoate metabolism	15	2	6.84×10^{-2}	1.17	0.15	0.11
Synthesis and degradation of ketone bodies	5	1	7.56×10^{-2}	1.12	0.15	0.60
Valine, leucine and isoleucine degradation	40	1	7.56×10^{-2}	1.12	0.15	0.00

[1)] Total Cmpd, The total number of compounds in the pathway.

[2)] Hit, The actually matched number from the user uploaded data.

[3)] FDR, The *p*-value adjusted using False Discovery Rate.

[4)] Impact; The pathway impact value calculated from pathway topology analysis.

Table 4. Pathway analysis of significantly different urine metabolites compared with healthy and subclinical ketosis group

Metabolic Pathway	Total Cmpd[1)]	Hits[2)]	*p*-value	-Log (*p*-value)	FDR[3)]	Impact[4)]
Ubiquinone and other terpenoid-quinone biosynthesis	9	1	4.02×10^{-3}	2.40	1.33×10^{-2}	0.00
Alanine, aspartate and glutamate metabolism	28	1	4.47×10^{-3}	2.35	1.33×10^{-2}	0.00
Selenocompound metabolism	20	1	4.47×10^{-3}	2.35	1.33×10^{-2}	0.00
Aminoacyl-tRNA biosynthesis	48	1	4.47×10^{-3}	2.35	1.33×10^{-2}	0.00
Valine, leucine and isoleucine degradation	40	1	4.52×10^{-3}	2.34	1.33×10^{-2}	0.01
Valine, leucine and isoleucine biosynthesis	8	1	4.52×10^{-3}	2.34	1.33×10^{-2}	0.00
Pentose phosphate pathway	22	2	5.17×10^{-3}	2.29	1.33×10^{-2}	0.05
Tyrosine metabolism	42	2	7.41×10^{-3}	2.13	1.67×10^{-2}	0.06
Starch and sucrose metabolism	18	1	1.47×10^{-2}	1.83	2.95×10^{-2}	0.07
Primary bile acid biosynthesis	46	1	3.33×10^{-2}	1.48	6.00×10^{-2}	0.02
Tryptophan metabolism	41	1	4.57×10^{-2}	1.34	7.48×10^{-2}	0.00

[1)] Total Cmpd, The total number of compounds in the pathway.

[2)] Hit, The actually matched number from the user uploaded data.

[3)] FDR, The *p*-value adjusted using False Discovery Rate.

[4)] Impact, The pathway impact value calculated from pathway topology analysis.

Figures

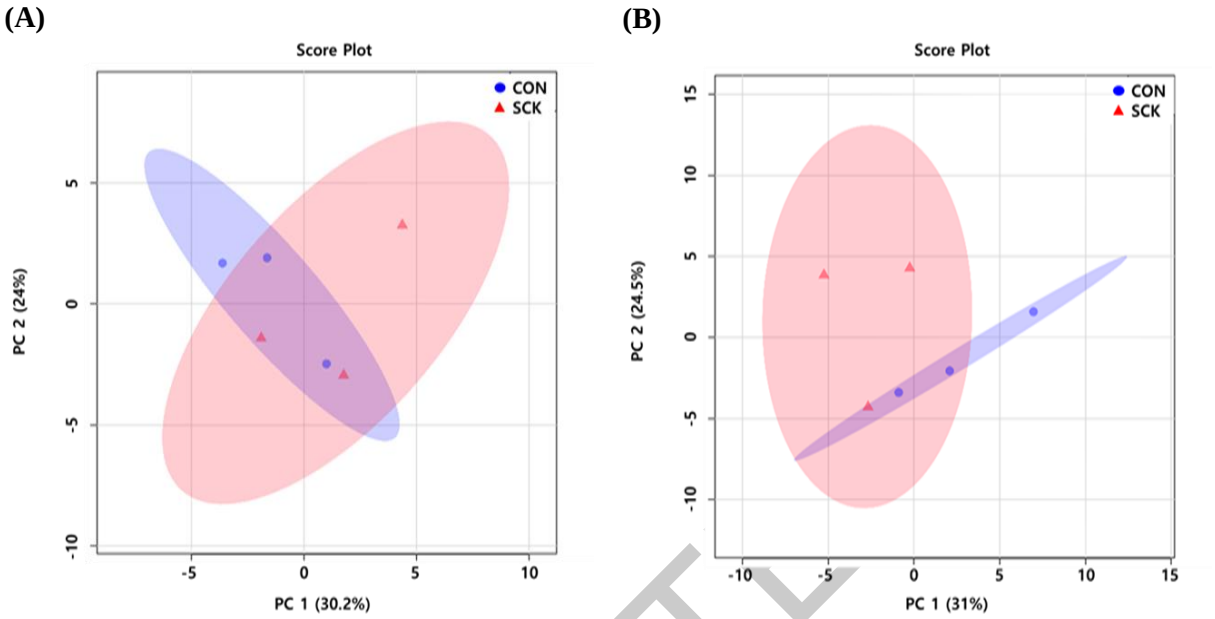

Fig 1. Principal components analysis score plot based on serum (A) and urine (B) metabolites data in healthy (CON) group and subclinical ketosis (SCK) and by proton nuclear magnetic resonance spectroscopy analysis.

On the score plot, each point represents an individual sample, with the blue dot representing the CON group (n=3), and the red triangle representing the SCK group (n=3). The abscissa and represent the variance associated with PC 1 and 2, respectively.

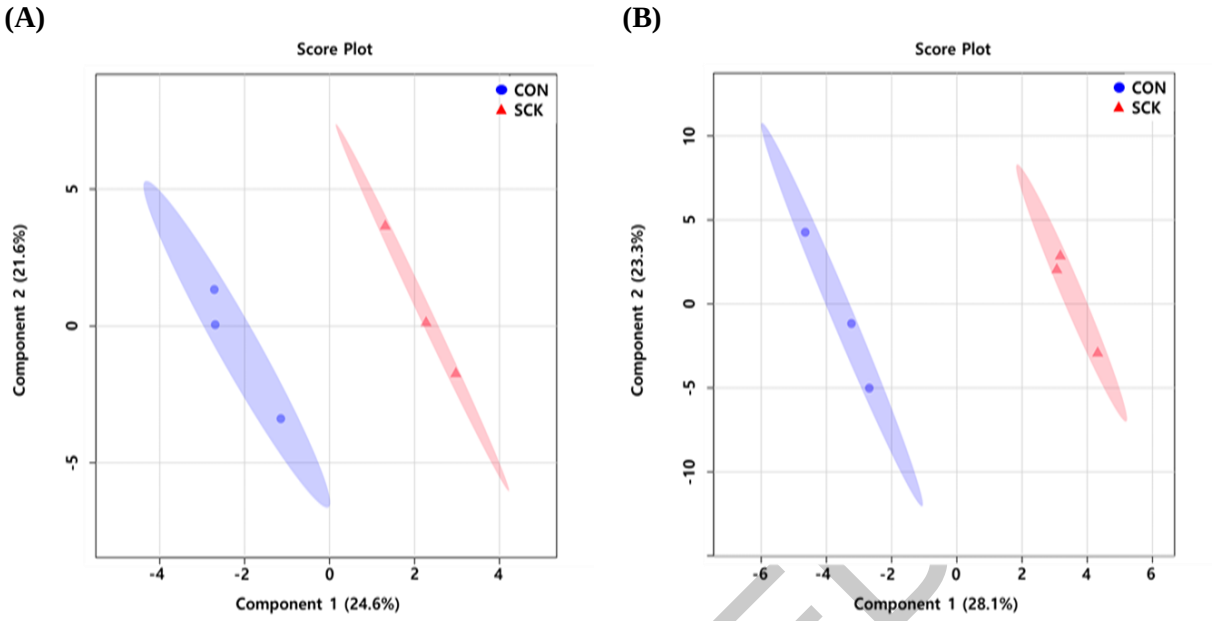

Fig 2. Partial least square-discriminant analysis score plot of serum (A) and urine (B) with healthy (CON) and subclinical ketosis (SCK) group by proton nuclear magnetic resonance spectroscopy analysis.

The shaded ellipses represent the 95% confidence interval estimated from the score. On the score plot, each represents an individual sample, with blue dot representing the CON group (n=3), and red triangle representing the SCK group (n=3) The abscissa and ordinate represent the variance associated with component 1 and 2, respectively.

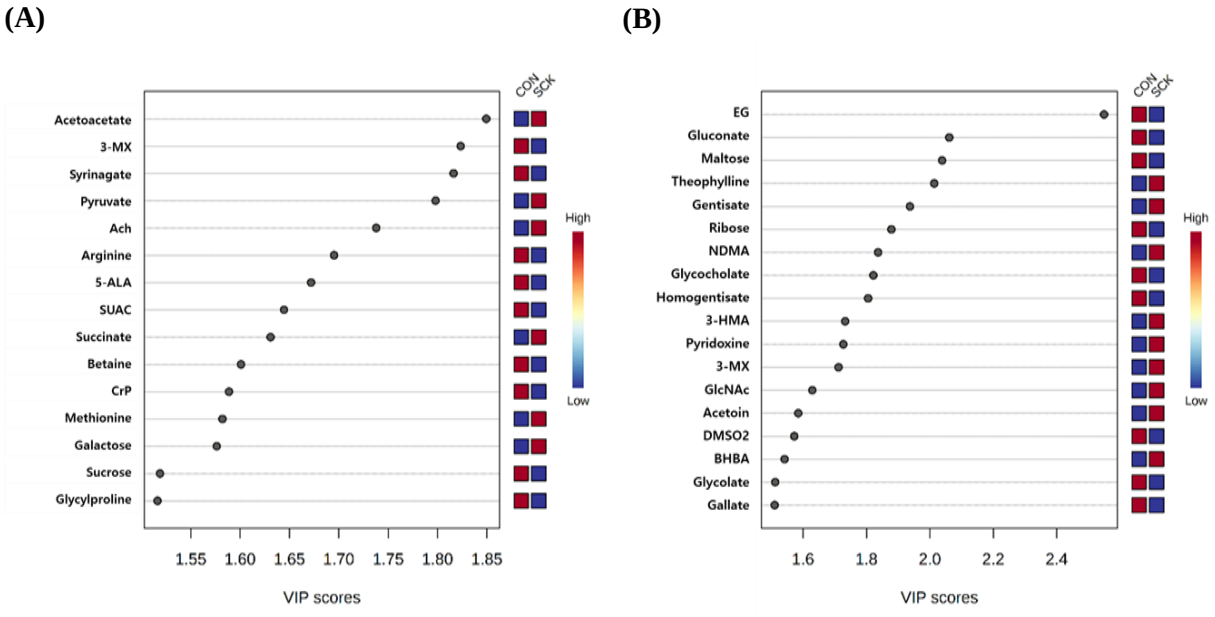

Fig 3. Variable importance in projection (VIP) scores of serum (A) and urine (B) metabolites in healthy (CON) group and subclinical ketosis (SCK) by proton nuclear magnetic resonance spectroscopy analysis. The selected metabolites were those with VIP score > 1.5. Heat map with red or blue boxes on the right indicates high and low abundance ratio, respectively, of the corresponding serum and urine metabolites in CON and SCK group. The VIP score was based on the partial least square-discriminant analysis model.

Serum metabolites VIP score value: Acetoacetate, 1.8494; 3-MX, 1.8235; syringate, 1.8163; ACh, 1.7379; arginine, 1.6952; 5-ALA, 1.6719; SUAC, 1.6445; succinate, 1.6309; betaine, 1.601; CrP, 1.5888; methionine, 1.5821; galactose, 1.5764; sucrose, 1.5189; glycylproline, 1.5164.

Urine metabolites VIP score value: EG, 2.5494; gluconate, 2.0611; maltose, 2.0386; theophylline, 2.0136; gentisate, 1.937; ribose, 1.8785; NDMA, 1.8365; glycoholate, 1.8216; homogentisate, 1.8051; 3-HMA, 1.7327; pyridoxine, 1.7268; 3-MX, 1.7118; GlcNAc, 1.6289; acetoin, 1.5846; DMSO2, 1.5721; BHBA, 1.5414; glycolate, 1.5119; gallate, 1.5102.

Metabolites abbreviation: 3-MX, 3-methylxanthine; ACh, O-acetylcholine; SUAC, succinylacetone; CrP, creatine phosphate; EG, ethylene glycol; NDMA, *N*-nitrosodimethylamine; 3-HMA, 3-hydroxymandelate; GlcNAc, *N*-acetylglucosamine; DMSO2, dimethyl sulfone; BHBA, 3-hydroxybutyrate.

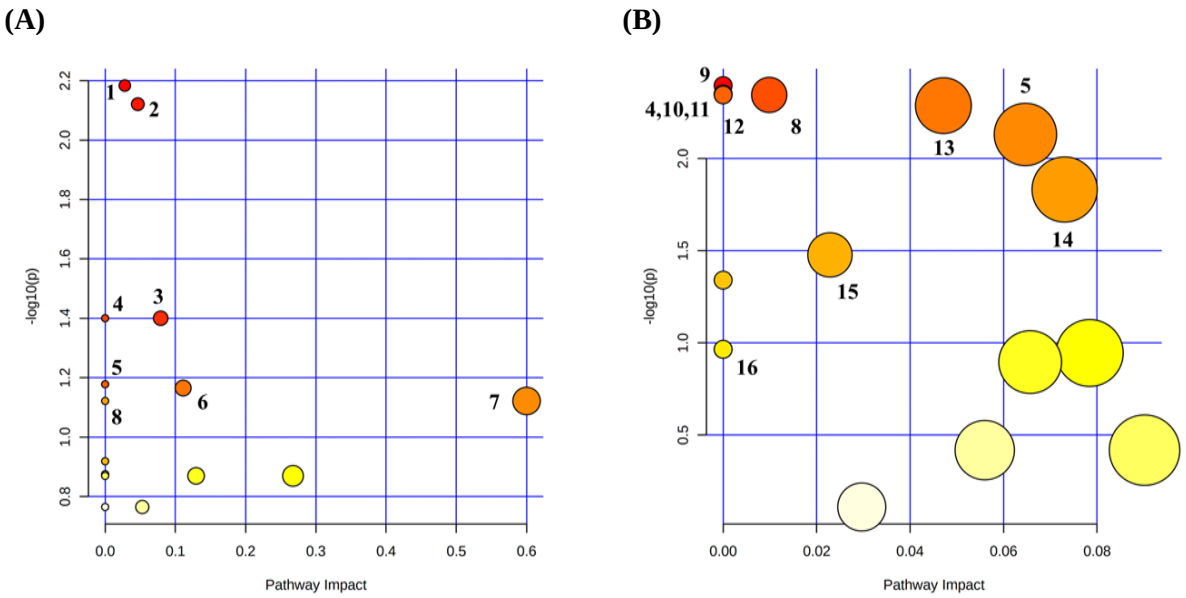

Fig 4. Metabolic pathway mapping significantly different serum (A) and urine (B) metabolites compared in healthy and subclinical ketosis group. The pathway impact analysis was performed using Metaboanalyst 5.0 software. The x-axis represents the pathway impact, and y-axis represents the pathway enrichment. The results are presented graphically as a bubble plot. The darker color and larger size represent higher *p*-value from enrichment analysis and greater impact from pathway topology analysis, respectively.

Metabolic pathway name: 1, Porphrin and chlorophyll metabolism; 2, Glycine, serine and threonine metabolism; 3, Citrate cycle (TCA cycle); 4, Alanine, aspartate and glutamate metabolism; 5, Tyrosine metabolism; 6, Butanoate metabolism; 7, Synthesis and degradation of ketone bodies; 8, Valine, leucine, and isoleucine degradation; 9, Ubiquinone and other terpenoid-quinone biosynthesis; 10, Selenocompound metabolism; 11, Aminoacyl-tRNA biosynthesis; 12, Valine, leucine and isoleucine biosynthesis; 13, Pentose phosphate pathway; 14, Starch and sucrose metabolism; 15, Primary bile acid biosynthesis; 16, Tryptophan metabolism.